PHD

The Role of Epithelial Cell De Differentiation in the Context of Improved Chemotherapy Applied to Pancreatic Cancer

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The Role of Epithelial Cell De-Differentiation in the Context of Improved Chemotherapy Applied to Pancreatic Cancer

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Abstract

In cancer, epithelial cell de-differentiation is a feature of rapidly dividing cells under non-controlled growth and it often reflects a change in the gene expression pattern; however, the relationship between proliferation and alterations in cellular differentiation has not yet been identified. This work examined how changes in the characteristics of cells that discriminate their differentiated and proliferative states can be used to improve on current pancreatic cancer chemotherapeutic strategies.

PepT1, a high substrate-capacity and low-affinity transporter system, has been suggested as an attractive drug delivery target for pancreatic cancer. Through a combination of immunological assays, PepT1 normally restricted to the apical surfaces in polarised intestinal epithelial cells, was shown to distribute at the cell membrane of non-polarised cancerous ductal cells. Anti-inflammatory or anti-cancer agents, like ibuprofen or gemcitabine, were conjugated to selected amino acids to enhance their uptake via PepT1. Studies with these conjugates demonstrated enhanced uptake into pancreatic cancer cells, AsPc-1 and HPAFII. Subsequent studies investigated how cell polarity that is typically disrupted in cancer can be modulated to affect the balance of epithelial differentiation *versus* proliferation. Pharmacological attenuation of YAP (c-Yes associated protein) using a β-adrenergic agonist, dobutamine, increased functional tight junction (TJ) structures and diminished proliferation rates of two pancreatic cancer cells, AsPc-1 and HPAFII. Dobutamine also primed an apoptotic cell response. When given in combination with gemcitabine, dobutamine further reduced cell proliferation.

Overall, these studies have provided support for using PepT1 as a method to target pancreatic cancer cells for the delivery of anti-cancer agents. Additionally, dobutamine was identified as a potential pharmacological agent to suppress the proliferation of pancreatic cancer cells by altering increasing cell programming that drives epithelial cell differentiation.

Abbreviation list

ABC	ATP-binding cassette
AJ	Adherens junctions
AJC	Apical junctional complex
AMPS	Ammonium Persulphate
APC	Apical polarity complex
ASPP	Apoptosis-stimulating p53 protein
BCH	2-Amino-2-norbornanecarboxylic acid
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
Cox	Cyclooxygenase
CP	Chronic pancreatitis
CPC	Crumbs polarity complex
CRB3	Crumbs family member 3
CTGF	Connective tissue growth factor
DAB	3, 3’ – diaminobenzidine
DAG	Diacyl glycerol
DAPI	4',6-diamidino-2-phenylindole
Dlg	Disks large
DMSO	Dimethyl sulfoxide
dsRNA	Double-stranded RNA
EC_{50}	Half maximal effective concentration
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunoabsorbent assay
EMT	Epithelial to mesenchymal transition
ESCC	Esophageal squamous cell carcinoma
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
fMLP	Formyl-Met-Leu-Phe
FR	Folate receptor

GFAP	Glial fibrillary acidic protein
GI	Gastrointestinal
GLUT1	Glucose transporter
GPCR	GTP-binding protein coupled receptor
HDAC	Histone deacetylase
HIAR	Heat induced antigen retrieval
HIV	Human immunodeficiency virus
HPDE	Human pancreatic ductal epithelial cell line
hPepT1	Human Peptide Transporter 1
Hpo	Hippo pathway
HRP	Horseradish peroxidase
IB	Ibuprofen
IBMX	3-isobutyl-1-methylxanthine
IFN γ	Interferon γ
IHC	Immunohistochemistry
IP_3	Inositol triphosphate
JAM	Junction adhesion molecule
LATs	System L amino acid transporters
LAT1	Large neutral amino acid transporter
LATS	Large tumor suppressor
Lgl	Lethal giant larvae
MARVEL	MAL and related proteins for vesicle trafficking and membrane link
miRNA	MicroRNA
MLC	Myosin light chain
MMP^1	Matrix metalloproteinases
MMP^2	Mitochondrial membrane potential
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2--(4-sulfophenyl)-2H-tetrazolium)
NSAIDs	Non-steroidal anti-inflammatory agents
Ocln	Occludin
PanIN	Pancreatic intra-neoplasia
PATJ	Protein associated with tight junctions
PBS	Phosphate buffered saline
PCP	Planar cell polarity
PDAC	Pancreatic ductal adenocarcinoma

PEG	Polyethylene Glycol
PepT1	Peptide transporter 1
PepT2	Peptide transporter 2
PFA	Paraformaldehyde
P-gp	P-glycoprotein
PHT	Peptide histidine transporter
PI	Propidium Iodide
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PP	Pancreatic polypeptide cells
PSC	Pancreatic stellate cells
PTR	Proton transporter
RISC	RNA-induced silencing complex
RNAi	RNA interference
ROCK	Rho kinase
SDS	Sodium dodecyl sulfate
Sdt	Stardust
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SLC	Solute carrier
TAZ	Transcriptional co-activator with PDZ-binding motif
TBS	Tris buffer saline
TEAD	TEA-domain family member
TEMED	Tetramethylethylenediamine
TER	Trans-epithelial electrical resistance
TGF-β	Transforming growth factor β
TJ	Tight junction
TNFα	Tumour necrosis factor α
TNM	Tumour, nodes and metastasis
Tric	Tricellulin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WT	Wild type
YAP	Yes-associated protein

ZO	Zonula occludens
ZO-1	Zona occludens protein-1
δ-ALA	Aminolevulinic acid

Chapter 1

1 Introduction

1.1 Pancreatic cancer

1.1.1 Microscopic Structure and Functional Activity of the Pancreas

The pancreas is a retroperitoneal organ surrounded by connective tissue that separates the parenchyma in lobules containing nerves, excretory ducts, lymphatic and blood vessels. Anatomically, the pancreas lies in the abdominal cavity behind the stomach (Figure 1.1). It is an endocrine gland and comprises two functional units that regulate two major physiological processes. The exocrine function assists in digestion and in the absorption of nutrients in the small intestine while the endocrine is primarily responsible for glucose metabolism.

The exocrine pancreas has two main components: acinar cells and pancreatic ducts. The acini are rounded structures formed by 40-50 pyramidal-shaped cells (Rovasio, 2010) (Figure 1.2-b,c). Centroacinar cells are spindle-shaped cells resulting from an extension of the intercalated duct cells into each pancreatic acinus. The acinar lumen is separated from the intercellular space by zonula occludens, ZO (Figure 1.2 – d). Polarised gland cells are characterized by the presence of filamentous mitochondria, a highly developed endoplasmic reticulum and an extensive population of polyribosomes (Rovasio, 2010) (Figure 1.2 – c). The exocrine function of the pancreas is stimulated by food ingestion through neuro-hormonal signals. This secretory activity is reflected on the number of zymogen granules: high number during fasting and scarce number after food ingestion (Rovasio, 2010). After the secretory vesicles fuse with the plasma membrane, they release their pro-enzymatic contents by exocytosis and its membrane becomes part of the acinar cell plasma membrane. These functional units cumulatively produce around 1200 mL of pancreatic juice daily that contains digestive enzymes such as trypsin, chymotrypsin, lipase and amylase. In turn, this enzyme mixture travels through a series of ducts to reach the duodenum lumen. Along this route, intercalated ducts introduce bicarbonate that feed into

intra-lobular ducts which then converge to the main pancreatic duct having a wider lumen and being limited by a single layer of cubic epithelium. The main ducts of the pancreas are formed by cuboidal epithelium delimited by a consistent *lamina propria*.

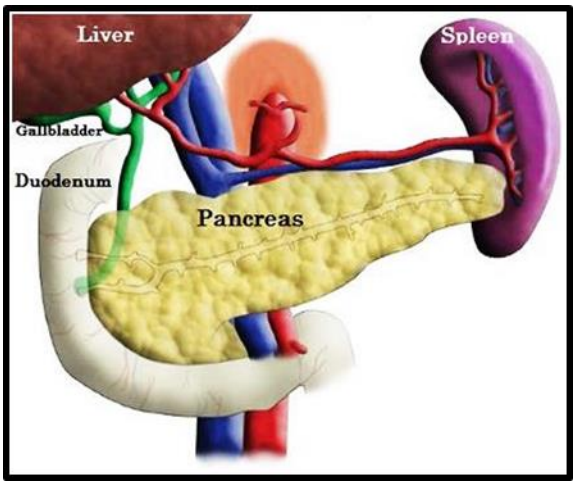


Figure 1.1 Anatomical position of the pancreas (Sircus, 2010).

In humans, pancreatic secretory activity is regulated directly through the effect of hormones produced in the islets of Langerhans on the blood flow and indirectly through the effect of the autonomic nervous system on the blood flow. The islets of Langerhans are hormone-producing structural units and are scattered into the gland parenchyma resembling a light immunohistochemistry staining compared to the surrounding acinar cells (Figure 1.3 - A). Each small cluster of cells is separated from the surrounding pancreatic tissue by a thin fibrous connective tissue layer. Hormones produced in these functional units are secreted by at least five types of cells: alpha cells produce glucagon (15-20 % of total islet cells), beta cells produce insulin and amylin (65-80 %), delta cells are responsible for somatostatin production (3-10 %), pancreatic polypeptide (PP) cells produce pancreatic polypeptide (3-5 %) and finally epsilon cells produce ghrelin (1 %). Beta cells are coupled electrically to other beta cells and islets can communicate with each other through paracrine and autocrine regulation (Rovasio, 2010).

Introduction

Branches of the mesenteric and celiac superior arteries, splenic and hepatic arteries, form the pancreatic vasculature. The lymphatic ganglia next to the splenic artery are responsible for the primary lymphatic drainage, whereas the secondary comes from the aortic, celiac, duodenal and sub-pyloric ganglia. Parasympathetic and sympathetic branches of the autonomic nervous system are responsible for the pancreas innervation. The sympathetic fibers are involved in vascular innervation and pain transmission while the parasympathetic fibers regulate the pancreatic exocrine secretion (Eynard, 2008).

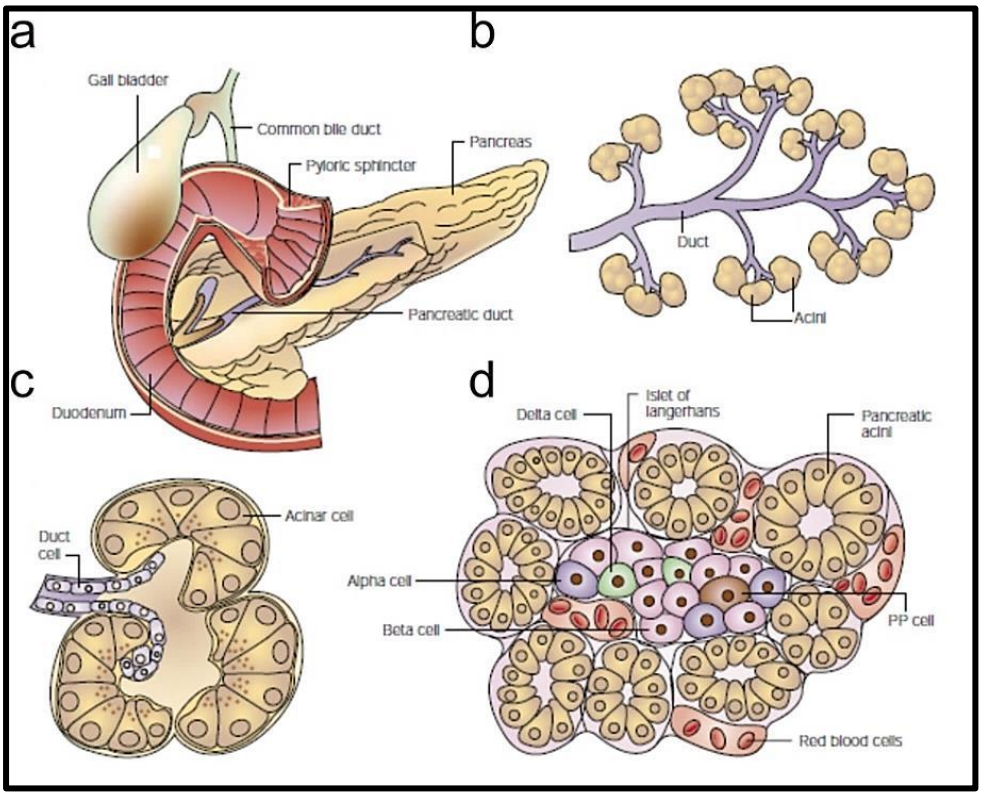


Figure 1.2 Schematic representation of the pancreas (a) Anatomy of the pancreas; **(b)** The exocrine pancreas; **(c)** Acinar and ductal cells arranged in a single acinus; **(d)** Exocrine compartment and Islet of Langerhans. Taken and reprinted by permission from Macmillan Publishers Ltd: Nat Rev Cancer (Bardeesy and DePinho, 2002), copyright, 2002.

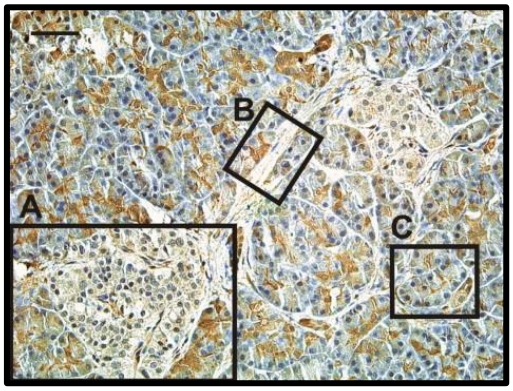


Figure 1.3 Tissue section from normal human pancreas. Biopsy sample stained for YAP, Hippo signaling main effector (Sudol, 1994). Scale bar, 50 µm. **(A)** Islets of Langerhans; **(B)** Pancreatic duct; **(C)** Acinar cell.

1.1.2 Development and structure of the pancreas

The stomach, duodenum, biliary system, including the gallbladder, liver and pancreas are derived from closely related structures in early embryogenesis. The pancreatic gland starts to develop at week 5 of gestation with dorsal and ventral endodermic invaginations from the most caudal segment of the primitive foregut (Figure 1.4 - Formation). When the duodenum grows and rotates around its longitudinal axis, the organs enlarge and the ventral pancreas together with the common bile duct rotates. Then, the inferior part of the pancreas head fuses with the dorsal bud during the 6 to 7th week of gestation forming the pancreas gland (Figure 1.4 – Rotation). In most cases, after 8 weeks of gestation, the pancreatic duct from the dorsal pancreas fuses with the pancreatic duct from the ventral pancreas to form the main pancreatic duct (Figure 1.4 – Fusion). After fusion, the pancreatic and biliary secretions travel through the ventral pancreatic duct gaining access to the duodenum.

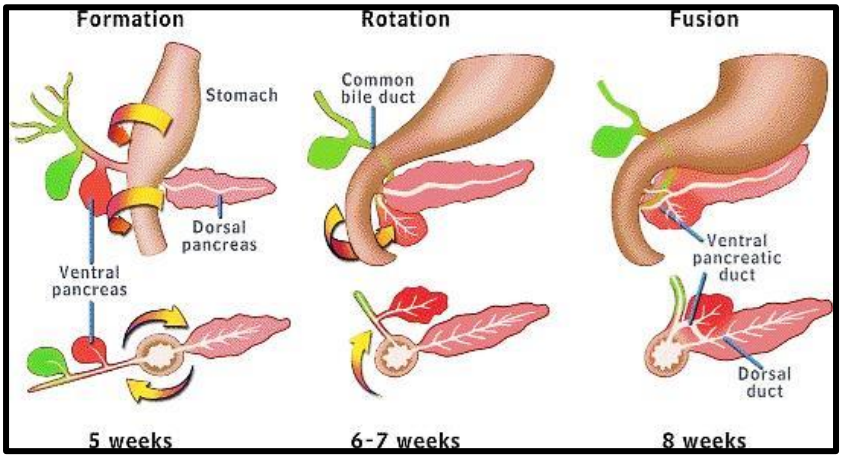


Figure 1.4 Structure and development of the pancreas. Taken and reprinted with permission from Morgan & Claypool Life Sciences (Pandol, 2010).

1.1.3 The nature of pancreatic cancer

More than 10 % of the total pancreatic cells form the pancreatic ducts. The best-characterized diseases of the exocrine pancreas are pancreatitis and cancer. It was not until the 19th century that the pancreas started to be appreciated as a vital organ. Nowadays, pancreatic cancer is recognized as one of the deadliest types of epithelial cancers. Pancreatic ductal adenocarcinoma (PDAC), commonly referred as pancreatic cancer, is a tumour type with ductal-cell histology and is by far the most common type of pancreatic neoplasms, accounting for more than 85 % of pancreatic cancers (Hruban et al., 2001). Pancreatic cancer carries one of the worst prognosis in the medicine field as it has a 5-year survival rate of only 3 % and a median survival of less than 6 months (Warshaw and Fernandezdelcastillo, 1992). Of greater concern is the statistics showing that these survival rates have not changed over the past 40 years.

PDAC is a very aggressive type of tumour as it grows rapidly; due to its anatomical position it readily metastasizes to lymph nodes and liver (Basturk et al., 2010). Typical symptoms of

PDAC include abdominal pain and jaundice due to tumour infiltration in nerves and the bile duct, however these are very vague (Erkan et al., 2013). Owing to the lack of specific symptoms, most of which are very subtle, allied to the limitations in diagnostic methods, pancreatic cancer often develops unperceived and is usually identified during a later, rather than early stage.

1.1.4 Epidemiology

There are approximately 277,000 annual cases of pancreatic cancer, of which 266,000 are deaths, corresponding to a 96 % mortality rate (Pandol et al., 2012). It is the eighth or ninth most frequent cause of cancer-related deaths worldwide and the fourth or fifth in developed countries (Maisonneuve and Lowenfels, 2010). Worldwide, pancreatic cancer occurs more often in men than in women and in urban areas more than in rural places (Jiao and Li, 2010). Patients diagnosed with pancreatic adenocarcinoma are usually between 60 and 80 years old and the mean age is around 63 (Evans, 2001).

In 2008, it was estimated that 37,700 patients in the US were diagnosed with PDAC of which 34,300 patients died from the disease (Jemal et al., 2008). In 2012, there were an estimated 43,920 new cases of pancreatic cancer of which 37,390 died (Siegel et al., 2012). In 2014, predictions point to 46,420 new cases with estimated death in 39,590 of these (Siegel et al., 2014) (Figure 1.5). Quite clearly the death toll as a consequence of this lethal malignancy is increasing. Despite the extensive efforts that have been made to improve the targeting of therapies to pancreatic cancer, none of these approaches have been shown to provide clinical value. In sum, the dismal prognosis for PDAC results from the combination of issues that are magnified by a lack of biomarkers that allow for early and effective screening,

resulting in a late diagnosis associated with metastatic dissemination of cancer cells that are noted for their resistance to systemic therapies (Feig et al., 2012).

An effective prevention measure in pancreatic cancer relies on well-defined risk factors by epidemiological research. Age and cigarette smoking are a well-established risk factor; accumulating evidence suggests obesity and diabetes as another possible risk factor (Jiao et al., 2009). However, the associations of alcohol, pancreatitis, dietary factors, infection agents as well as occupational and hormonal factors and pancreatic cancer are yet to be confirmed (Jiao and Li, 2010) (Table 1.1). Pooled and large prospective studies have emerged to provide epidemiological evidence on risk factors of pancreatic cancer, suggesting that by adapting a healthy life style, pancreatic cancer can be prevented in almost 50 % of the cases (Jiao et al., 2009).

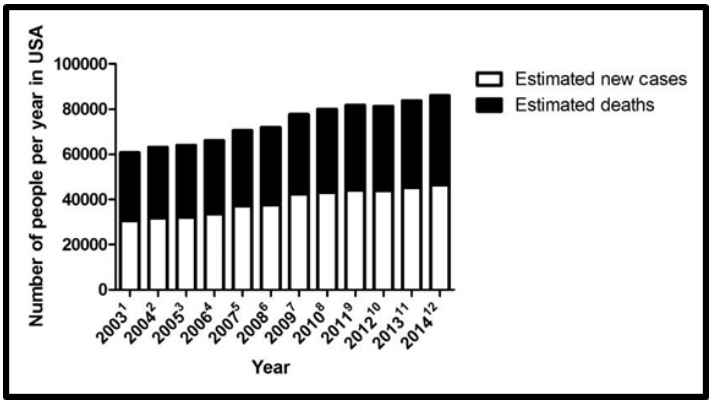


Figure 1.5 Number of estimated new cases and deaths per year due to PDAC in USA [1] - (Jemal et al., 2003); [2] - (Jemal et al., 2004); [3] - (Jemal et al., 2005); [4] - (Jemal et al., 2006); [5] - (Jemal et al., 2007); [6] - (Jemal et al., 2008); [7] - (Jemal et al., 2009); [8] - (Jemal et al., 2010); [9] - (Jemal, 2011); [10] - (Siegel et al., 2012); [11] - (Siegel et al., 2013); [12] - (Siegel et al., 2014)

Table 1.1 Established and suspected risk factors of pancreatic cancer.

Established risk factors	Suspected risk factors
Age	Alcohol
Cigarette smoking	Chronic pancreatitis
Hereditary	Dietary factors
Obesity	Infectious agents
Diabetes Mellitus	Occupational and hormonal factors

1.1.5 Pathologic classification and biological behavior of pancreatic neoplasia

Pancreatic neoplasms are classified according to their characteristics. Some of these classifications include acinar cell carcinoma, ductal adenocarcinoma, endocrine tumours, solid pseudo-papillary tumours, lymphoplasmacytic sclerosing pancreatitis and primary pancreatic lymphoma (Mortenson et al., 2008). Studies have described other histological variants of pancreatic cancer comprising medullary carcinoma, hepatoid carcinoma, adenosquamous carcinoma and undifferentiated carcinoma (Degen et al., 2008).

The endocrine pancreas is responsible for producing a variety of hormones but unlike the exocrine component, which releases enzymes locally to reach the duodenum, the hormones produced here are secreted into a rich capillary network that is associated with each islet. Endocrine cancers are far less common than ductal neoplasms, accounting for only 1 to 2 % of all pancreatic tumours (Basturk et al., 2010). Most of these neoplasms can be detected early as most of them secrete one or more active peptides that produce clinical symptoms (D'Onofrio et al., 2004).

Despite the fact that acinar cells contribute to the vast majority of pancreatic tissue, acinar cell carcinoma is quite rare, representing only 5 % of all exocrine tumours (Tobias, 2010). Macroscopically, tumours formed by cancer cells derived from acinar cells are solid, well circumscribed; they occasionally display necrosis, hemorrhage or cystic characteristics. Acinar cancer cell tumours are well demarcated, having a higher rate of surgical resection (64 %) compared to ductal adenocarcinoma (Klimstra and Adsay, 2009).

Although the ductal system is not a complex structure, it is the source of the majority of neoplasms in the pancreas, around 90 % (Klimstra and Adsay, 2009). This propensity

toward malignant transformation is probably due to many factors, one of which has suggested to result from the fact that the ductal system is the only component in the pancreas exposed to the outside environment, increasing its potential exposure to mutagens (Basturk et al., 2010). PDAC will be the focus of the remainder of this chapter.

1.1.6 Invasive ductal neoplasia

As previously mentioned, the ducts transport the acinar enzymes to the duodenum (Figure 1.6); in order to be protected and lubricated, mucins form a stable network lining the pancreatic ducts (Basturk et al., 2010). Ductal differentiation can form different types of structures, which give the name to some of the resulting tumours. Tubular units (lumen forming), papilla (finger-like projections of the mucosa lining the ducts) and cysts are hallmarks of ductal differentiation. It has been speculated that PDAC originates not only in ductal cells but also in acinar (Guerra et al., 2007), islet (Pour, 2003) and centro-acinar cells (Stanger et al., 2005).

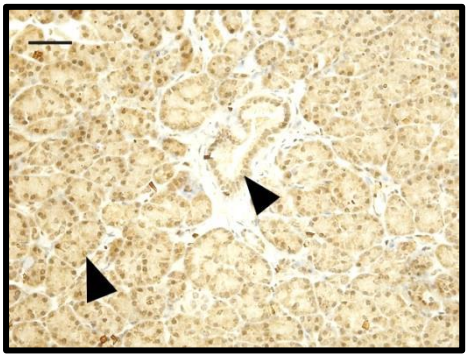


Figure 1.6 Tissue section from normal pancreas. Biopsy sample stained for Slug, a highly conserved zinc finger transcription repressor (Hemavathy et al., 2000). Acinar cells are arranged in lobules forming the majority of the parenchyma. As part of their normal secretory function, the nuclei are polarised to the periphery of the cells and arranged in round units forming the acinus (pointed by the black arrows). In the center, an intra-lobular duct lined by cuboidal-low epithelium can also be observed (pointed by the black arrow); Scale bar, 50 µm.

More than 85 % of pancreatic tumours are invasive ductal adenocarcinomas, also designated as scirrhous, tubular or pancreatobiliary type (Von Hoff et al., 2005). In general, these are all very aggressive cancers with only 20 % of the cases being considered for surgical resection at the time of diagnosis (Evans, 2001). Rapid growth, insidious infiltration and early dissemination contribute to the extremely low cure rate of PDAC (Evans, 2001) (Figure 1.7). Around 80 % of PDAC spread through nerves and vessels resulting in a phenomenon called perineural invasion, which is thought to be the reason for back pain, one of the most common symptoms of this tumour (Basturk et al., 2010). Vascular invasion is also very common in ductal neoplasia as cancer cells are capable of forming well delineated glandular structures in vascular spaces (Basturk, 2008) (Figure 1.8 – b).

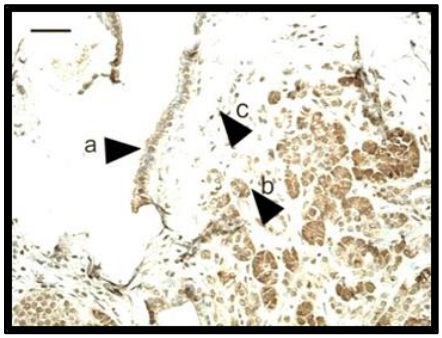


Figure 1.7 Tissue section from a patient diagnosed with invasive ductal neoplasia. Biopsy sample stained for PepT1, an oligopeptide transporter (Fei et al., 1994). The ducts **(a)** start to change morphologically and are no longer well defined. Acinar cells **(b)** are no longer polarised and are diluted in the highly intense fibrotic stroma **(c)**. As the neoplasm becomes more evident, cells start to be pleomorphic, tall and columnar, with hyperchromatism and an abundant cytoplasm; Scale bar, 50 µm.

Figure 1.8 Tissue section from a patient diagnosed with low differentiated ductal neoplasia with vascular invasion. Biopsy sample stained for YAP, Hippo pathway main effector molecule (Sudol, 1994). Ductal cells demonstrating a low grade of differentiation **(a)** and vascular invasion **(b)** are identified. Scale bar, 50 µm.

The diagnosis of PDAC can be quite problematic, not only from a clinical point of view but also microscopically. PDAC is characterized by a prominent desmoplastic response (Figure 1.7), in which a dense stroma makes it difficult to distinguish from inflammatory lesions of this organ, in particular types of chronic pancreatitis (CP) (Basturk et al., 2010). From the microscopic point of view, injured native ducts from non-inflamed or non-cancerous pancreas can show substantial cytologic atypia comparable to that of ductal carcinomas; most ductal adenocarcinomas form well differentiated glandular structures that resemble benign ducts or that cause ductal obstruction leading to CP (Adsay and Zamboni, 2004).

1.1.7 Non-invasive ductal neoplasia

Pancreatic intra-epithelial neoplasia (PanIN) is the most common precursor lesion of PDAC, being first described in 1998 (Brat et al., 1998). These abnormal intra-ductal proliferations that often accompany invasive ductal adenocarcinoma may also be seen in the absence of PDAC (Klöppel et al., 1980). Microscopically, PanINs are characterized by nuclear enlargement and deposition of abnormal nuclear material also designated as

hyperchromasia. Altered cellular metabolism leads to the accumulation of genetic abnormalities including p53 mutations, glycoproteins, mainly mucins, loss of cell polarity and increased mitotic activity. The spectrum of changes is classified into different categories: PanINs 1,2 and 3 (Figure 1.9). PanIN1 are present in 70 % of normal pancreas at autopsy and are characterized by flat epithelial lesions composed of tall columnar cells with small nuclei, round to oval and may demonstrate mutations in the GTPase known as K-Ras. PanIN2 are papillary mucinous epithelial lesions with some nuclear abnormalities such as loss of polarity, nuclear crowding and hyperchromasia. PanIN3 are papillary or micropapillary but rarely flat and show loss of nuclear polarity, dystrophic goblet cells, abnormal mitoses, nuclear irregularities and prominent nuclei (Hruban et al., 2001) .

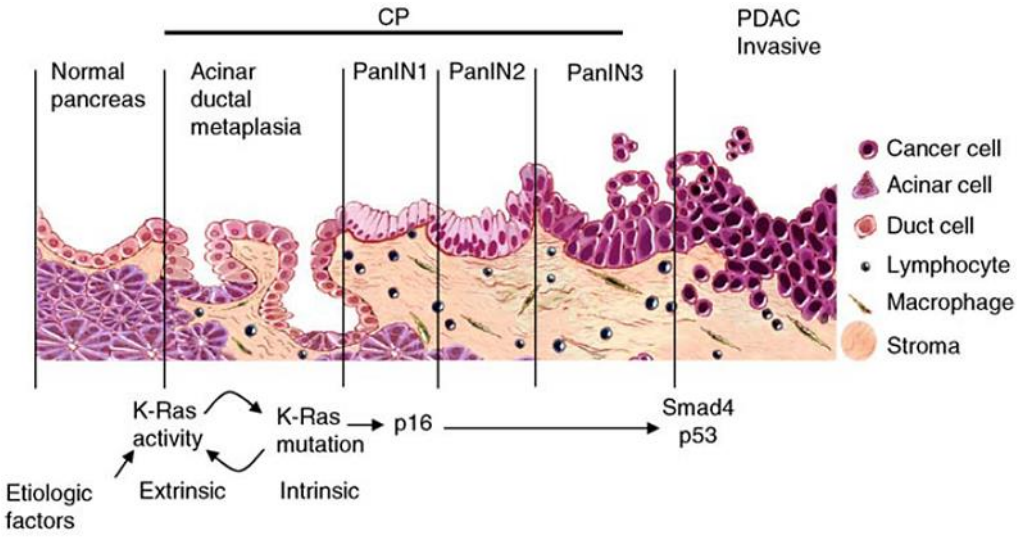


Figure 1.9 Schematic diagram showing the molecular and anatomical evolution from non-invasive to invasive PDAC. Grading of precursor lesions, PanINs, comprising a neoplastic transformation ranging from early mucinous change (PanIN1) to frank *carcinoma in situ* (PanIN3). Taken and reprinted with kind permission from Springer Science and Business Media (Logsdon et al., 2010).

1.1.8 Molecular relationship between chronic pancreatitis and pancreatic cancer

The association between inflammation and cancer has been well defined in some organs (Mantovani et al., 2008). Nevertheless, the distinction between CP and PDAC is considered one of the most, if not the most, challenging differential diagnosis in pathology. The relationship between CP and PDAC is well established, although it remains controversial whether CP is a precursor or an independent pathology unrelated to PDAC (Logsdon et al., 2010). Both pathological conditions share similar molecular traits such as the presence of stroma, inflammation with leucocyte infiltration, acinar cell atrophy and distorted and blocked ducts (Witt et al., 2007). Equally, the extent of fibrosis increases with the progression of the lesions and PanINs are present in most patients with CP alongside chromosomal instability and genomic damage, similarly to PDAC (Figure 1.9). CP provides a microenvironment that contains dendritic cells, activated leukocytes including macrophages, neutrophils, mast and T cells which secrete a wide variety of cytokines, chemokines and enzymes associated with the production of reactive oxygen species (Pelicano et al., 2004).

K-Ras activity leads to the activation of NF-kB (Karin and Greten, 2005) and cyclooxygenase 2, Cox-2 (Harris, 2007), which is a major transcription factor that regulates genes responsible for both the innate and the adaptive immune response. Both K-Ras and NF-kB appear to be activated in CP; it has been suggested that they potentially provide the link between inflammation and PDAC (Rakonczay et al., 2008; Schlosser et al., 2002). Further evidence has suggested that treatment increasing K-Ras activity produces inflammation associated with fibrosis and accelerates the progression of PDAC (Guerra et al., 2007) and that K-Ras alone may be sufficient to trigger both of these pathological conditions (Logsdon and Ji, 2009). Thus, it is likely that K-Ras activity is a common underlying mechanism between CP and PDAC. Nonetheless, most of the patients

diagnosed with CP, do not progress to PDAC, hence critical barriers are likely to exist between the mechanisms involved in CP development and the onset of PDAC (Logsdon et al., 2010).

Chronic inflammation of the pancreas leads to irreversible deterioration of the organ structure and function. CP presents a rather challenging diagnosis as it occurs in many forms from different etiologies. From a histological perspective, CP is characterized by loss of parenchyma, extensive fibrosis and by the presence of inflammatory cells (Witt et al., 2007) (Figure 1.10). Despite the primary clinical manifestation of fibrosis, CP events appear to initiate in the acinar cells (Logsdon et al., 2010). Although it is common to identify regions that would be defined as CP within histological sections of PDAC, this association does not define a required dependent mechanism between CP and PDAC.

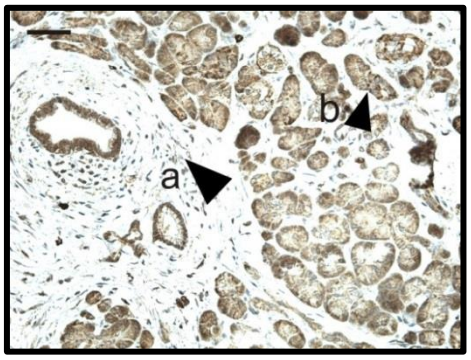


Figure 1.10 Tissue section from a patient diagnosed with CP. Biopsy sample stained for c-Yes, non-receptor tyrosine kinase. Presence of an intense fibrotic response **(a)** and loss of normal parenchyma characterized by acinar cell displacement imbedded in an abundant stroma **(b)**. Scale bar, 50 µm.

Different etiological factors are involved in the development of CP; alcohol consumption, autoimmune cystic fibrosis, heredity, tropical and idiopathic forms and gallstone are contributing factors to the pathology (Witt et al., 2007). It has been reported that CP appears to increase the risk of developing PDAC and hereditary pancreatitis has been found to be associated with mutations in the trypsinogen gene leading to dysfunctional digestive function

(Whitcomb, 2004). Hereditary pancreatitis has been reported to increase the risk of PDAC by 53-fold with a cumulative lifetime risk of approximately 40 % (Lowenfels et al., 1997). Nevertheless, the mutations observed in hereditary CP are not the same as those detected in PDAC (Whitcomb, 2004).

1.1.9 The cell cycle and pancreatic cancer

In 2000, Hanahan and Weinberg outlined six biological properties acquired during the multistep development of human tumours: self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, reduced apoptosis, sustained angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg, 2000) (Figure 1.11). In 2011, conceptual progress has added two more emerging hallmarks: reprogramming of energy metabolism and evading immune destruction (Hanahan and Weinberg, 2011) (Figure 1.11). Deregulated cell proliferation is one of the hallmarks of cancer (Hanahan and Weinberg, 2011); PDAC cells display high levels of proliferating activity, indicative of altered cell cycle regulation. Tuveson and co-workers were able to show that the proliferation rate in early PanIN lesions was 18-fold higher relative to normal pancreatic ducts (Hingorani et al., 2003). Amongst the plethora of oncogenic events and genetic alterations occurring in PDAC, mutations in the K-Ras oncogene are the most frequent. These genetic alterations are present in a significant fraction of early precursor lesions suggesting the involvement of K-Ras in PDAC initiation (Moskaluk et al., 1997). The tumour suppressor p16 is lost in almost all PDAC cases (Schutte et al., 1997). Loss of p53 and SMAD4 tumour suppressor proteins has also been reported (Sun et al., 2001; Tascilar et al., 2001) (Figure 1.12). This section will focus particularly on K-Ras and SMAD4.

Mutations in the *K-RAS* oncogene have been reported to occur in approximately 90 % of PDAC patients (Zhou et al., 2004). Ras proteins activate signaling downstream effector pathways, the best studied of which are Raf-MAPK and PI3 Kinase-Akt (Downward, 2003). These signaling cascades are involved in driving proliferation, survival and cell growth; they have also been reported to prime the events of cellular transformation and oncogenesis (Lewis, 2010). The essential role(s) of various K-Ras-induced signaling pathways in the PDAC phenotype is currently unknown.

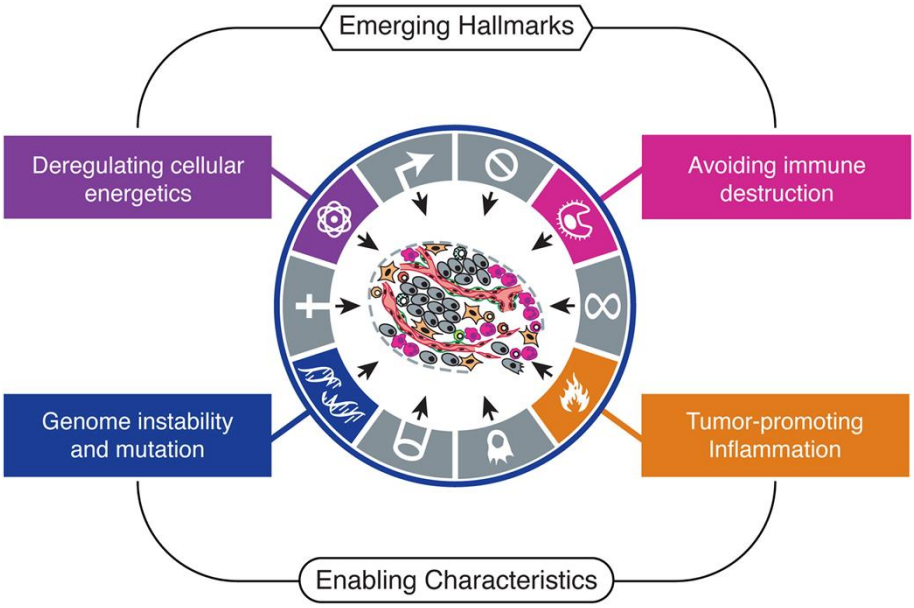


Figure 1.11 The next generation of the hallmarks of cancer (Hanahan and Weinberg, 2011). Taken and reprinted with permission from Elsevier.

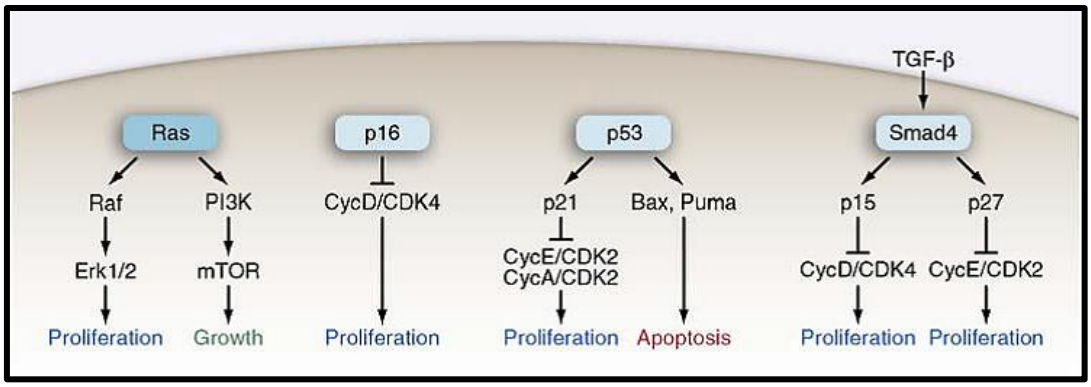


Figure 1.12 Genetic alterations occurring in pancreatic cancer. A schematic illustration of how genes commonly altered in pancreatic cancer affect cell cycle progression. Taken and reprinted with kind permission from Springer Science and Business Media (Lewis, 2010)

The SMAD pathway is the canonical signaling pathway that the transforming growth factor (TGF-β) family members signal through (Figure 1.12). This complex signaling network regulates cellular proliferation, apoptosis, cell migration and ultimately cell invasion. TGF-β ligands bind to type I or II receptors and such interactions induce receptor dimerization, activation and subsequent cellular signaling. Activated TGF-β receptors then recruit SMADs that bind to SMAD4, which will translocate to the nucleus inducing the regulation of different target genes. TGF-β signaling is inactivated in pancreatic cancer, leading to an excessive activation of pancreatic cancer cell growth. Nearly 50 % of PDAC have lost the *SMAD4* gene, whereas only a fraction show inactivation of TGF-β receptor (Hahn et al., 1996). *SMAD4* mutations are not present in early PanIN lesions but do appear in PanIN3, suggestive of a role in malignant conversion of precursor lesions into adenocarcinoma (Lewis, 2010).

1.1.10 Emerging therapeutic targets for pancreatic cancer and tumour microenvironment

Despite numerous efforts, targeted therapies have only provided limited efficacy in treating patients suffering from PDAC. More than 80 % of patients are diagnosed in the final and advanced metastatic stage, excluding the possibility of a surgical resection (Hotz et al., 2007). At this stage, PDAC tumours are highly metastatic and show a vigorous stromal response, termed desmoplasia, which significantly restricts the successful penetration of agents designed to target these tumours. These tumours are characterized by an increased rate of deregulated proliferation, enhanced cell survival, resistance to therapy, evasion from immune control, and increased metastasis (Feig et al., 2012) (Figure 1.13 – b). The tumour mass that is produced in PDAC commonly consists of a dynamic assortment of cells that include pancreatic stellate cells (PSCs), lymphatic and vascular endothelial cells, immune

cells and fibroblasts. This complex cellular environment is dynamic, changing in its composition during the progression from early precursor lesions PanIN to invasive PDAC.

In normal pancreas, PSCs are in a relative quiescent state and localize close to the acinar cells, capillaries and nerve fibers and can be identified by expression of GFAP (glial fibrillary acidic protein) and desmin (Erkan et al., 2013; Omary et al., 2007) (Figure 1.13-a). In normal pancreas, PSCs account for 4-7 % of all parenchymal cells, whereas in PDAC they can outnumber the cancer cells (Yen et al., 2002). PSCs have been reported to be the major source of excessive deposition of extracellular matrix proteins such as collagens, fibronectin and proteoglycans in the pancreatic cancer stroma (Bachem et al., 1998). This prominent and dense fibrotic stroma impacts on pancreatic cancer aggressiveness, as well as providing a physical scaffold with an intensive production of cytokines and growth factors, creating a physical barrier to the tumour (Erkan et al., 2013). The fibrotic stroma causes an extremely hypoxic environment leading to distortion and compression of tissue vasculature and ultimately prevents efficient drug perfusion (Erkan et al., 2007). PSCs have also been shown to actively secrete matrix metalloproteinases (MMP[1]), which are associated with inflammation, fibrosis, cancer invasion and ultimately angiogenesis (Fujisawa et al., 2012). Thus, it is possible that therapeutic benefit may be gained through strategies that deplete PSCs.

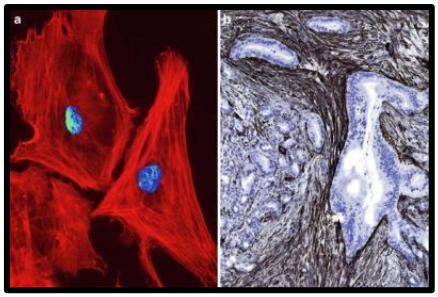


Figure 1.13 Tumour microenvironment in pancreatic cancer. (a) Immunofluorescence analysis of cultivated primary pancreatic stellate cell; **(b)** immunohistochemistry analysis of PDAC biopsy sample, hematoxylin-eosyn staining, original magnification: 100x; Taken and reprinted with kind permission from Springer Science and Business Media (Erkan et al., 2013).

1.1.11 Targeted therapies in pancreatic cancer

Significant progress has recently been achieved in the treatment of many tumour types such as colorectal cancer, lung and melanoma, but a comparable success has not yet been achieved for pancreatic cancer (Yauch and Settleman, 2012). Gemcitabine, a commonly used chemotherapeutic agent in treating PDAC, produced only a modest increase in patient survival rate (Burris et al., 1997). Different approaches for targeted therapies have demonstrated huge success in a pre-clinical context; however they have largely failed in the clinical setting, suggesting the inability of pancreatic tumour models to recapitulate critical aspects and/or barriers associated with the tumour microenvironment in patients. This chapter section will focus on some of the most promising of these therapeutic strategies: anti-angiogenic, EGF (epidermal growth factor) inhibition, K-Ras inhibition and MMP[1] inhibition.

TKI258, a tyrosine kinase inhibitor to vascular endothelial growth factor receptor, VEGF-R, delayed growth and improved survival in subcutaneously injected tumours and orthotopic models (Taeger et al., 2011). Another study reported pre-clinical benefit in using anti-VEGF-R2 antibodies covalently bound to doxorubicin in depleting VEGF-R2-expressing tumour vasculature (Wicki et al., 2012). Another study highlighted the potential benefit of gemcitabine given in combination with bevacizumab, a humanized monoclonal antibody that blocks specifically vascular endothelial growth factor receptor A, VEGF-RA (Kindler et al., 2010). Despite the fact that these successful pre-clinical approaches have led to clinical trials, there was a great discrepancy between experimental results and clinical reality.

In terms of EGF inhibition, the EGF receptor (EGFR) tyrosine kinase erlotinib, commercialized under the name Tarceva (Genentech), when combined with gemcitabine, increased the overall survival from 5.91 to 6.24 months, which translates to approximately

10 days (Moore et al., 2007). Despite its cost, the overall survival advantage is minimal. Additionally, a study has reported that combining gemcitabine with the combination therapy known as FOLFIRINOX (composed of the vitamin B derivative folinic acid, the pyrimidine analogue 5-fluorouracil (5-FU), the topoisomerase inhibitor irinotecan, and the platinum-based antineoplastic oxaliplatin) produced a significant survival benefit of 11.1 vs 6.7 months in patients with metastatic PDAC compared to gemcitabine as a single treatment (Conroy et al., 2011b). Still, due to its increased toxicity, FOLFIRINOX is only suitable for some patients (Feig et al., 2012). Recently, data published from a phase II trial, in which gemcitabine was given in combination with oxaliplatin and cetuximab, lacked significant improvements (Merchan et al., 2012).

K-Ras mutations have been reported in 90 % of patients suffering from PDAC (Zhou et al., 2004). Still, in a study evaluating the efficacy of cetuximab plus gemcitabine/oxaliplatin in pancreatic cancer patients with the *K-RAS* gene mutated, neither monotherapy nor combined approaches showed a clinical benefit (Kullmann et al., 2011).

Given the role of MMPs[1] in pancreatic tissue maintenance (Fujisawa et al., 2012), MMP[1] inhibitors have emerged. Ukrain, a potent MMP[1] inhibitor, was shown to down-regulate MMP[1]-2 and MMP[1]-9 in pancreatic cancer cell lines and was also able to decrease invasion (Funel et al., 2010). Yet, this approach has also failed in the clinic (Conroy et al., 2011a).

Since conventional and targeted therapies largely failed in prolonging survival in pancreatic cancer patients, new approaches and novel strategies are needed to improve the clinical outcome for PDAC patients.

1.1.12 Membrane transports as therapeutic targets in cancer

Nearly 4 % of the genes in the human genome encode for membrane transporters, underlying the importance and varied roles played by these proteins (Venter, 2001). Membrane transporters function on the selective uptake of nutrients, nucleosides, amino acids, inorganic ions and sugars (Venter, 2001). Transporter proteins can be classified into two major families, solute carrier (SLC), which comprises both facilitated and secondary active transporters and the ATP-binding cassette (ABC) transporters. While the family of ABC transporters is very extensive and functionally highly diverse, all members of this family utilize the energy generated from ATP hydrolysis.

Various drugs can be substrates for specific membrane transporters, including anti-cancer drugs; these interactions can affect absorption, distribution and excretion pathways. Some membrane transport proteins, however, might also be capable of internalizing anti-cancer drugs in a pro-drug form that emulates an endogenous substrate. Thus the use of specific transporters as molecular targets in cancer therapy has been discussed (Cravo and Mrsny, 2013) (Table 1.2). The remaining portion of this chapter will focus on three of these drug transporters: folate, glucose and PepT1 transporter with a special reference to PepT1 as it is the drug delivery target subject of this thesis.

The folate receptor (FR) is overexpressed at the cell surface of ovarian cancer cells (Weitman et al., 1992), the most lethal gynecologic malignancy (Kim et al., 2012). Based on this, FR represents a particularly “smart” approach for targeting chemotherapeutics into cancer cells. Another important feature in using FR as a cancer target is the small size of the targeting ligand, which allows for repeated administration with a less likely immunogenic scenario. Additionally, there is the possibility in delivering therapeutic agents through

cytosolic endocytosis (Sudimack and Lee, 2000). Many efforts have been developed through the use of liposomes in therapy; nonetheless, major limitations underpin the use of immune-liposomes for drug delivery. Apart from the immunogenicity of the targeting ligand, the covalent attachment of a protein to a liposome is technically demanding (Sudimack and Lee, 2000). Lee and Low entrapped doxorubicin, a cancer drug commonly used to treat leukemia as well as cancers of the bladder, breast, stomach, lung and ovaries, in folate-PEG-liposomes and were able to show a 45- to 86-fold higher uptake and cytotoxic action in cancer cells compared to the free drug (Lee and Low, 1995). However, in cancer, the total amount of FR throughout the body is much greater than that expressed in a specific tumour; hence drug-folate conjugated therapies have the potential to induce systemic toxicity.

As a polar molecule, glucose cannot get into cells via simple diffusion. The GLUT1 glucose transporter is responsible for the facilitated transport of glucose into cells (Macheda et al., 2005). Although constitutively expressed in normal tissues, the GLUT1 transporter has attracted some attention as it can be overexpressed in many types of tumours (Brown and Wahl, 1993; Yamamoto et al., 1990). Further, cancer cells are typically more dependent than non-cancer cells upon glycolysis compared to mitochondrial oxidative phosphorylation to produce ATP (Abouzeid et al., 2013). A recent study has reported a dramatic improvement in efficacy between the non-targeted and GLUT1-targeted forms of curcumin, and doxorubicin-loaded micelles in a model of colorectal carcinoma (Abouzeid et al., 2013). Thus, GLUT1 was identified as a potential novel strategy worth further clinical investigation.

Table 1.2 List of some transporter systems able to shuttle specific anti-cancer drugs (Cravo and Mrsny, 2013).

Transporter system name		Endogenous substrate	Type of transport	Anticancer drug
Organic anion: OATP1A2		Bile salts, organic anion and cation	Facilitated transport	Methotrexate
Organic cation:	OCT1	Organic cation polyspecific	Facilitated transport	Oxaliplatin, Glivec®
	CT2	Carnitine, betaine	Facilitated transport	Doxorubicin
Folate		Folate	Exchanger/OH^-	Methotrexate, Tomudex®, edatrexate
Glucose: GLUT1		Glucose	Facilitated transport	18-FDG
Peptide: PepT1		Oligopeptides	Cotransporter/H^+	Bestatin

Abbreviations: *18-FDG* 18 fludeoxyglucose

1.2 The oligopeptide transporter system PepT1

1.2.1 Structure and function

PepT1, also known as solute carrier family 15 member 1 (SLC15A1) belongs to the proton-coupled oligopeptide transporter family, PTR, which also includes PepT2 (SLC15A2), peptide histidine transporter 1 (PHT1, SLC15A4) and peptide histidine transporter 2 (PHT2 , SLC15A3) (Steiner et al., 1995). From this family, only PepT1 and PepT2 have been shown to have transport activity in humans (Liang et al., 1995).

PepT1 uses an electrochemical proton gradient as the driving force (Figure 1.14). The optimal extracellular pH for substrate uptake is between 4.5 and 6.5, with compounds that have no net charge at this pH being taken most efficiently.

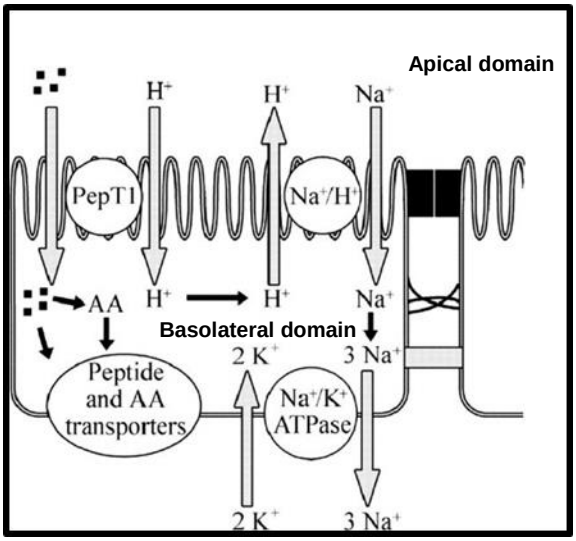


Figure 1.14 PepT1 transport. Peptide transport through PepT1 is coupled to an inward proton gradient to catalyze electrogenic uptake against a concentration gradient. The sodium anti-porter pump, NH3, located at the apical region, exports protons entering the cell through PepT1 in exchange for Na^+ ions. Adapted from Gilbert (Gilbert et al., 2008).

In 1994, Fei and co-workers first cloned and characterized PepT1 by expressing PepT1 cRNA in *Xenopus laevis* oocytes (Fei et al., 1994). One year later, PepT1 was isolated from humans (Liang et al., 1995). PepT1 consists of 708 amino acids and 12 putative membrane domains and has a core molecular mass of 79 kDa (Liang et al., 1995).

PepT2 is composed of 729 amino acids and is located at the luminal membrane of the distal part of the proximal tube in the kidney and shares about 50 % amino acid identity with PepT1. PepT1 shares a similarity of 83 % between humans (Liang et al., 1995) and mice (Fei et al., 2000). The main role of PepT1 is the intestinal absorption of nutrients; conversely PepT2 has a role in the reabsorption of peptide associated amino nitrogen from the glomerular filtrate (Leibach and Ganapathy, 1996). As a consequence of the distinct physiological roles, PepT1 is a low affinity but high capacity transport system whereas PepT2 is a high affinity but low capacity transport system (Leibach and Ganapathy, 1996; Liang et al., 1995). Both PepT1 and PepT2 have been extensively studied and reviewed

regarding their transport mechanisms, tissue distribution and regulation, substrate specificity and structural characteristics.

1.2.2 Substrate conformational and structural requirements

The crystal structure for PepT1 is not yet available due to the technical challenge in crystalizing protein membranes; nevertheless site-directed mutagenesis has identified some amino acid residues such as Y12, Y56, Y64, Y91, Y167, H57, W294 and E595 to be important in modulating substrate binding and transport activity (Brandsch et al., 2008). In terms of structural substrate requirements, there is a preference for L- relative to D- amino acids, and for peptides with an acidic or hydrophobic function at the C-terminus and a weakly basic group at the N-terminus (Brodin et al., 2002). Nearly all di/tri-peptides can be transported by PepT1, although neither free nor tetra-oligopeptides can be transported through this system with great efficiency (Chen et al., 2002a). Transport of peptides and peptidomimetics occurs in a stereospecific manner and the transporter system discriminates substrates by differences in binding affinity that affects the maximal transport rate (Wenzel et al., 1995). The minimal substrate requirement involves two oppositely charged head groups separated by a carbon spacer, and a peptide bond is not essential in the substrate structure (Rubio-Aliaga and Daniel, 2008).

Being a high substrate capacity system capable of shuttling substrate molecules up to 600 Da, and because of its low affinity (Daniel, 1996; Steiner et al., 1995), PepT1 represents an attractive strategy for drug delivery. Many studies have explored the potentialities of PepT1 as a drug shuttling system. Examples of known substrates for PepT1 include β-lactam antibiotics such as cefadroxil (Ganapathy et al., 1995), the anti-herpes virus compound δ-aminolevulinic acid (δ-ALA) (Doring et al., 1998), the anti-parkinsonism dipeptide pro-drugs

L-α-methyldopa-L-Phe and L-dopa-L-Phe (Brandsch et al., 2008). More recently, the compound JBP485, a dipeptide with anti-hepatitis activity, was also shown to be a substrate for PepT1 activity (Liu et al., 2011).

Different methods can be used to screen PepT1 substrates. Through competitive inhibition with known PepT1 substrates, polarised monolayers of Caco-2 cells are frequently used as an *in vitro* cell model to assess whether a drug fulfills the requirements of a PepT1 substrate. Other cell lines besides Caco-2 have also been used to screen for PepT1 substrates such as HeLa cells, MDCK (Madin-Darby canine kidney cells) or CHO cells stably expressing PepT1. Because of its electrogenic features, the uptake through PepT1 can also be tested in *Xenopus laevis* oocytes induced to express PepT1 following the injection of cRNA encoding PepT1 (Fei et al., 1994). Genetically modified mice deficient in PepT1 are also available, representing a valuable strategy for screening compounds *in vivo* (Hu et al., 2008).

1.2.3 PepT1 regulation

PepT1 expression and function are under tight regulation that is controlled by numerous physiological, pharmacological and ultimately pathological factors. PepT1 undergoes diurnal regulation and fasting or starvation can up-regulate PepT1 expression both at mRNA and protein levels. While there is no known pathology associated with PepT1 malfunction at a genomic level, PepT1 up-regulation has been reported in inflammation (Buyse et al., 2002; Ingersoll et al., 2012; Vavricka et al., 2006; Wang et al., 2013). Pharmaceutical agents such as tacrolimus and cyclosporin (Motohashi et al., 2001), 5-FU (Inoue et al., 2005) as well as various hormones (Rubio-Aliaga and Daniel, 2008) have also been linked to regulate PepT1 expression.

1.2.4 PepT1 as a drug delivery target

In mammals, PepT1 is predominantly expressed in the small intestine; it localizes at the brush border of epithelial cells (Lu and Klaassen, 2006). The concept of targeting intestinal PepT1 for improved oral absorption and availability has been well explored (Brandsch, 2009; Doring et al., 1998; Ganapathy et al., 1995; Liu et al., 2011). PepT1 expression has been reported in cancer tissues fuelling interest in adopting PepT1 as a possible route of drug delivery to these types of cancer.

Gonzalez and co-workers reported the surprisingly high expression of PepT1 in two human pancreatic cancer cell lines, AsPc-1 and Capan2; with both cell lines having a ductal origin and a mutated K- Ras genotype (Gonzalez et al., 1998). This finding has driven the interest in adopting PepT1 as a possible drug delivery strategy to target pancreatic cancer cells specifically. Since pancreatic cancer cells overexpress this transporter system relative to levels observed in non-cancerous cells, this difference may allow for enhanced drug delivery to PDAC. Subsequent to the findings by Gonzalez and co-workers, increased levels of peptide transporter activity was reported in gastric cancer, osteosarcoma, bladder cancer and cholangiosarcoma (Inoue et al., 2005; Knutter et al., 2002). Additionally, bestatin, an anti-cancer drug shown to be a PepT1 substrate (Inui et al., 1992), was reported to have a much higher level of intracellular accumulation in a specifically constructed HeLa cell line stably expressing high levels of PepT1 compared to the normal cell line (Nakanishi et al., 2000).

1.3 Cell polarity in cancer

1.3.1 The main players in the establishment and maintenance of epithelial cell polarity

Epithelial tissues are distributed throughout the body to establish a lining that separates the external world from the internal surfaces of the body. Each epithelium has specialised functions related to its distinct location and physiological role(s). These specialised functions are largely dependent upon the structural organization of cells within those tissues regulating cell polarity. Epithelial cells exhibit apical-basal polarity defined by two different domains: the apical membrane facing the outside surface of the body and basolateral membrane oriented away from the lumen. Epithelial cells also display planar cell polarity (PCP), referring to the polarization of a field of cells within the lateral plane of a cell sheet (Jones and Chen, 2007).

Epithelial cell polarity mechanisms also constitute a barrier to metastasis through a close interaction with the apical junctional complex (AJC), which comprises TJ and adherens junction (AJ) structures. AJC elements have been shown to be regularly mutated in epithelial cancers, which reinforces the role of AJC complex in suppressing cancer malignancy (Royer and Lu, 2011). Extensive intracellular signaling networks comprising three protein complexes are involved in establishing and maintaining the polarization of an epithelium: Par, Crumbs and Scribble (Figure 1.15).

The PAR complex has been extensively studied in *Drosophila melanogaster*. The core of the PAR complex includes the PDZ-domain-containing proteins, PAR3 and PAR6 and an atypical protein kinase C zeta isoform, aPKC. PAR complexes have been shown to regulate

the formation and maintenance of apical-basal polarity in cells, however there are multiple isoforms of Par6 and aPKC denoting some functional redundancy (Aranda et al., 2008).

The Crumbs protein complex is a key regulator of epithelial polarity; being initially discovered in *Drosophila melanogaster* as an essential protein for maintaining apico-basal polarity and integrity of embryonic epithelia (Bulgakova and Knust, 2009). The core components of the Crumbs complex are Crb, Stardust (Sdt), PATJ (protein associated with TJ) and Lin-7 (Bulgakova and Knust, 2009). Formation of the Crumbs complex results from dynamic interactions with these core components. These elements are scaffolding proteins that serve as a platform that recruits other proteins to close proximity thereby facilitating their function as a complex. Studies have shown that reducing the expression levels of PATJ1, Pals1 or Lin-7 in MDCK cells leads to defects in TJ formation and consequently reduction of TER and abnormal cell polarity (Straight et al., 2004; Wang et al., 2007a).

The Scribble complex, including disks large (Dlg) and lethal giant larvae (Lgl), is critical for the establishment of the basolateral membrane domain. In contrast to PAR and Crumbs complexes, the role of the Scribble polarity complex is less understood. Nevertheless, several lines of evidence have demonstrated that mutations in any member of this complex results in a pronounced disorganization of epithelial architecture, which is characterized by loss of cell-cell adhesions (Bilder and Perrimon, 2000).

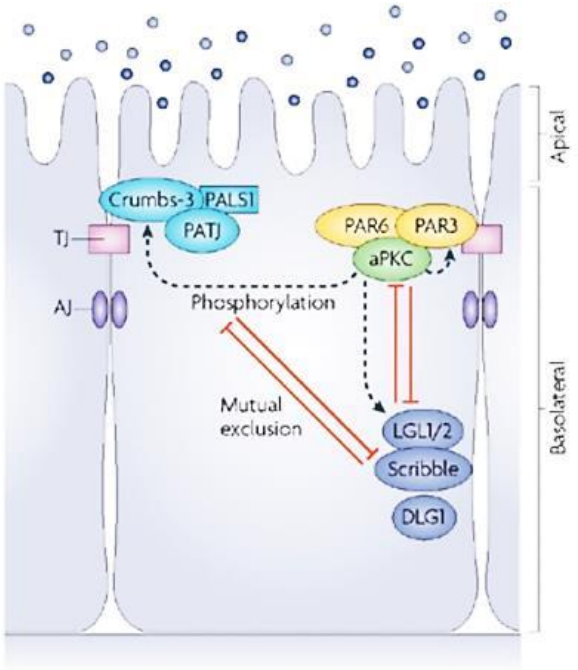


Figure 1.15 Proteins involved in the apical polarity complex (APC). Taken and reprinted with permission from Macmillan Publishers Ltd (Iden and Collard, 2008).

1.3.2 TJ and Ocln

Apical-basal polarity has two main fundamental roles in epithelial cells linked to tumour suppression: maintenance of the AJC and regulation of proliferation (Royer and Lu, 2011). TJs are the most apical structures and govern the permeability of confluent sheets of epithelial cells. TJs participate in many different processes, such as sealing the intercellular space, separation of apical and basolateral fluid compartments of epithelia and endothelia, barrier to cell migration and participation in cell-cell adhesion. Besides, TJs can also act as intermediates and transducers in cell signaling processes involved in polarity, cell differentiation, growth and proliferation (Martin and Jiang, 2009). These structures are mainly involved in two types of signaling: those that transduce information from the cell interior towards TJs to guide their assembly and regulate their function and those that are

transmitted to the cell interior to modulate the gene expression involved in proliferation or differentiation (Matter and Balda, 2003). The TJ network comprises five families of proteins: junctional adhesion molecules (JAMs), claudins, and MARVEL proteins that include occludin (Ocln), tricellulin (Tric) and MarvelD3 (Figure 1.16).

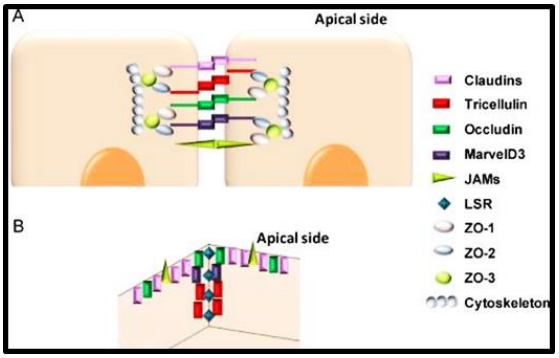


Figure 1.16 Schematic diagram showing protein distribution at TJs. (A) Bi-cellular TJ; **(B)** Tri-cellular TJ. Taken and reprinted from The Lancet with permission from Elsevier (Mariano et al., 2011).

JAMs are a group of proteins with a molecular weight of approximately 40 kDa involved in regulating a plethora of cellular adhesive processes including leukocyte migration (Martin-Padura et al., 1998), cell morphology (Mandell et al., 2005) and intercellular junction assembly (Liang et al., 2000). JAMs are subdivided into two subgroups according to their structure and sequence. JAM-A, JAM-B, JAM-C, also known as JAM-1, JAM-2, JAM-3 respectively, have a class II PDZ-binding domain at the C-terminus whereas JAM-4 contains a class I PDZ-binding motif at the C-terminus (Ichikawa-Tomikawa et al., 2011). JAM-A participates in the formation of TJs and polarity through interaction with Par-3/aPKC/Par-6 (Suzuki et al., 2002); JAM-B and JAM-C interact with Par-3 through PDZ binding domain (Ebnet et al., 2004) and JAM-4 associates with Ligand-of-Numb protein X1 (Kansaku et al., 2006) and MAGI-1 (Hirabayashi et al., 2003). In contrast to Ocln or claudin, JAM-related proteins do not form TJ strands, raising the possibility of having different biological roles than these other TJ components (Itoh et al., 2001).

Claudin belongs to a family comprising 24 distinct members. They are tetra-span proteins with a molecular weight of around 18 to 27- kDa (Tsukita et al., 2001). There are many isoforms generated by alternative splicing exhibiting different expression patterns and physiological functions (Van Itallie et al., 2006). Claudins constitute the TJ backbone, as they are able to reconstitute close membrane appositional structures in fibroblasts (Furuse et al., 1998). Claudins confer the major and structural features of TJs and their phosphorylation is associated with TJ assembly and function in regulating paracellular permeability (Ichikawa-Tomikawa et al., 2011). Two classes of claudins with different roles have been reported; Isoforms -4,-5,-8 and -11 and -19 have been linked to barrier sealing properties, whereas -2,-7,-10 and -15 have been demonstrated to have pore forming properties (Krause et al., 2008).

Ocln (~65 kDa) was the first integral membrane protein of TJ to be identified, being shown to localize at epithelial and endothelial TJs (Furuse et al., 1993). Ocln exhibits a multi-domain tetra-span structure and each individual domain has distinct functions (Figure 1.17). Ocln also exhibits a MARVEL (MAL and related proteins for vesicle trafficking and membrane link) domain which is a four-transmembrane structural motif involved in membrane apposition and fusion events (Cummins, 2012). The extended C-terminus has been shown to interact with ZO-1, zona occludens protein-1, mediating its trafficking to the plasma membrane. Approximately half of the Ocln amino acid residues localize to the C-terminus and increased serine phosphorylation has been shown to correlate with its localisation at TJs and paracellular barrier formation (Wong and Gumbiner, 1997). The C-terminus is also a target for multiple kinases including c-Yes and c-Src oncogene proteins that when activated, cell-cell junctions get dynamically reorganized (Chen et al., 2002b). The extracellular Ocln loops have been demonstrated to participate in its localisation and TJ stability (Balda et al., 2000).

A variety of studies have demonstrated Ocln to have a key role in the dynamic regulation of TJ permeability. McCarthy and co-authors reported that Ocln overexpression in MDCK cells enhanced trans-epithelial electrical resistance (TER) (McCarthy et al., 1996). Furthermore, down-regulation of Ocln via either proteolysis or through growth factors, such as VEGF, or phospholipase D2, was directly linked to increased endothelial permeability (Murakami et al., 2009). Past studies have also suggested the extent of Ocln down-regulation to correlate with tumour grade, invasiveness and metastasis (Wang et al., 2007b). Additionally, Li and Mrsny also showed the crucial role Ocln plays when TJs are disrupted by oncogenic Raf1 protein. Importantly, forced re-expression of Ocln into this system was able to reverse the phenotypical changes induced by active Raf1 namely loss of intercellular junctions, loss of an epithelial phenotype and ultimately the acquisition of an invasive phenotype (Li and Mrsny, 2000).

Tric is also a tetra-span protein that concentrates at tri-cellular contacts of epithelial cells (Ikenouchi et al., 2005). Like Ocln, Tric also displays MARVEL domains (Cummins, 2012); The MARVEL domain has been found to have a putative role in vesicle transport of proteins which is consistent with the observed vesicular trafficking of Ocln (Erickson et al., 2007). This group of proteins has been speculated to display a certain degree of redundancy contributing uniquely to TJ regulation and epithelial function. Genes encoding for both Ocln and Tric are located in chromosome 5 and it has been suggested they may have arisen from gene duplication during evolution (Ikenouchi et al., 2005). Tric is related to epithelial barrier and organization of both bi- and tri-cellular junctions. Moreover, Tric expression can be repressed by SNAI1, a zinc-finger type transcription factor that plays an important role in epithelial to mesenchymal transition, EMT (Dorfel et al., 2009). Recessive mutation of the *tricellulin* gene is linked to non-syndromic deafness (Riazuddin et al., 2006).

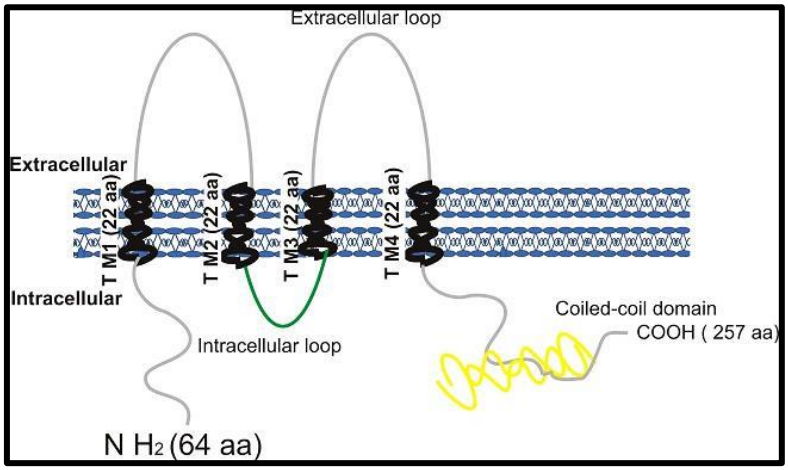


Figure 1.17 Ocln structure with regard to its organization within the plasma membrane.

1.3.3 Cell Polarity and EMT

EMT is an essential process occurring during embryogenesis, growth and wound repair by which epithelial cells lose their cell polarity and cell-cell-adhesion properties and gain the capacity to migrate and proliferate (Barrallo-Gimeno and Nieto, 2005). Pathological activation of EMT events leads to the initiation of uncontrolled growth and metastasis, which results in cancer formation and progression (Thiery, 2003). Several lines of evidence have suggested that loss of core polarity proteins could induce EMT. Given their potential role as tumour suppressors, core polarity proteins are often targeted and disrupted by oncogenic signaling (Royer and Lu, 2011). Studies have suggested that the deregulation of polarity proteins such as Scrib, Dlg and Lgl, triggers malignant transformation (Huang and Muthuswamy, 2010). Recent work suggested that cell polarity may limit growth through suppression of the Hippo (Hpo) pathway (Halder and Johnson, 2011).

1.3.4 The Hpo pathway signaling

The Hpo pathway, also known as the Salvador/Warts, is a conserved signaling pathway with a crucial role in regulating epithelial tissue growth, alongside cell proliferation, apoptosis and other stress responses, events that can be disrupted in epithelial cancers (Badouel et al., 2009). In contrast to other signaling pathways, such as EGF, TGF- β or WNT signaling pathways, the Hpo pathway does not have extracellular growth factor components for signaling, instead being regulated by upstream components involved in regulating cell adhesion and polarity (Johnson and Halder, 2014). Epithelial cell shape, adhesion and density appear to be directly regulated by the intracellular localisation and phosphorylation state of transcriptional regulators of the Hpo pathway known as YAP and TAZ (transcriptional co-activator with PDZ-binding motif). The protein kinases MST 1/2 and their regulatory protein, SAV1, are at the core of the Hpo signaling cascade and interact together to form an activated complex. RASSF family proteins can also activate MST 1/2 which can then directly phosphorylate LATS 1/2, large tumour suppressor homolog kinases (Dong et al., 2007). At high cell density, the WW-domain containing transcriptional co-activators YAP are phosphorylated at Ser^{127} and TAZ at Ser^{89}; 14-3-3 binds to YAP thereby inhibiting its translocation to the nucleus. At low cell density, YAP is mainly nuclear and YAP/TAZ serves as a co-activator for the TEA-domain family member (TEAD) group of DNA-binding transcription factors, which will then promote survival and proliferation programs (Dong et al., 2007; Zhang et al., 2008; Zhao et al., 2007) (Figure 1.18).

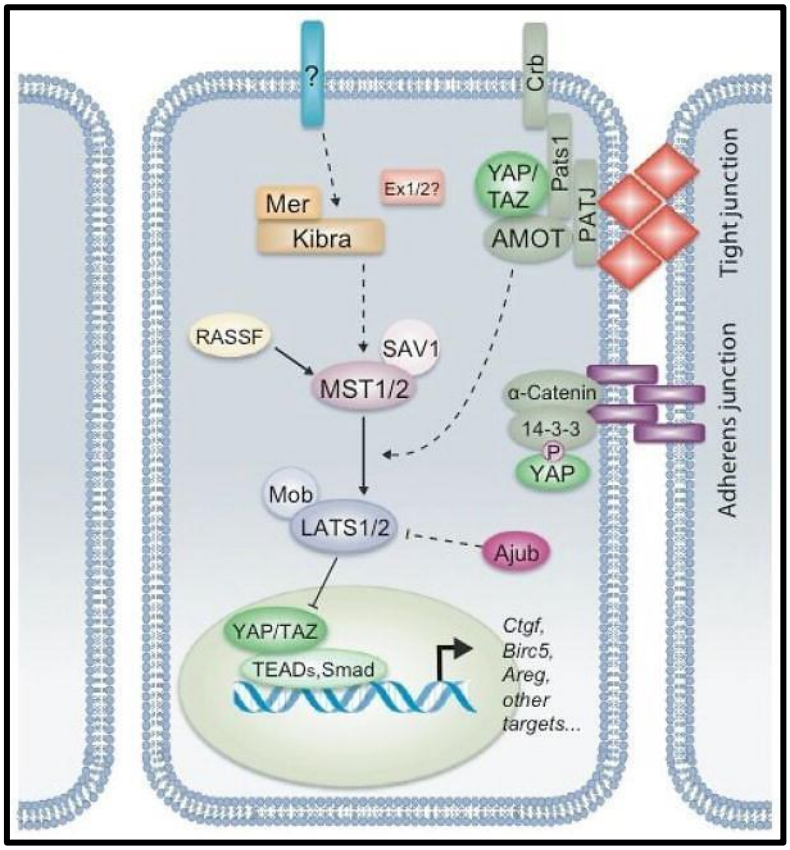


Figure 1.18 The Hpo pathway signaling cascade. Solid lines demonstrate known interactions and dashed lines unknown mechanisms. Blunted lines and arrows indicate either activation or inhibition. Taken and reprinted from The Lancet, with permission from Elsevier (Ramos and Camargo, 2012).

1.3.5 The Hpo pathway and apico-basal cell polarity

The apical domain and cell-cell junctions play a key role in Hpo pathway regulation. It is not yet known, however, what extracellular signals are sensed through cell-cell contacts via the Hpo pathway activation (Genevet and Tapon, 2011). Given the role of Hpo pathway in contact inhibition (Zhao et al., 2007), a speculative possibility is that TJ/AJs might function as a signaling receiving platform to sense through the Hpo pathway signals from neighbor cells. Translocation of YAP to the nucleus at high cell density, coincides with the deletion of AMOT-like proteins, PALS1 or CRB3 (Crumbs family member 3) (Varelas et al., 2010; Zhao et al., 2007). Thus, it appears that the Hpo pathway, and in particular YAP/TAZ interacts

with elements of the TJs to sense apical polarity and TJ organization (Schlegelmilch et al., 2011). While E-cadherin has been identified as a cell-cell contact sensor of AJs, no comparable cell-cell contact element for TJs has been identified.

1.3.6 The Hpo pathway and planar cell polarity

PCP represents an essential feature of organs and tissue that regulates their pattern and extent of controlled growth. During development, PCP mechanisms control the size and shape of cells that can then organize to form organs and tissues of consistent and reproducible size. The external epithelia of *Drosophila* is the tissue commonly used to study PCP defects (Simons and Mlodzik, 2008). Here, PCP appears to be controlled by two signaling pathways: Fz/Dsh (Frizzled/Dishevelled) and the Ft pathway (Klein and Mlodzik, 2005). The study of PCP in vertebrates has been hampered by redundancy as some isoforms do not have overlapping expression patterns (Simons and Mlodzik, 2008); double and triple knockouts are often the only way to address the study of this mechanism, albeit technically demanding. From these studies a concept has emerged.

The Hpo pathway can function as a sensor of epithelial integrity and architecture that can override proliferation stimuli involving ligand-receptor pathways. Hpo signaling cascades receive information for position inputs, mechanical forces and other stressors that reflect the state of the epithelia. These cues ensure a tightly controlled regulation of tissue growth during development and a mechanism to maintain tissue homeostasis in adult tissues (Simons and Mlodzik, 2008).

1.3.7 Regulation of cell proliferation and apoptosis by Hpo signaling

Understanding how cell proliferation and cell death are coordinated during tissue growth and homeostasis assumes vital importance in preventing diseases such as cancer (Huang et al., 2005). Deregulated YAP and TAZ can lead to enhanced cell proliferation and survival coupled to the acquisition of cancer phenotypes characterized by cancer stem cells characteristics, EMT, inhibition of senescence and drug resistance (Johnson and Halder, 2014).

In mammals, the core kinase components of the Hpo pathway are MST1/2 and LATS 1/2; both sets of kinases are regarded as important regulators of apoptosis. Growing evidence however, suggests YAP to have a dual role in both inhibiting and inducing apoptosis. As an apoptotic inducer, YAP activates p73, a member of the p53 tumour suppressor family, promoting the expression of pro-apoptotic elements such as bcl-2 family (Strano et al., 2005). As an apoptotic inhibitor, studies suggested ASPP (apoptosis-stimulating p53 protein) to inhibit the interaction of YAP with LATS1, blocking therefore YAP/TAZ phosphorylation, which in turn leads to nuclear accumulation of YAP/TAZ promoting cell migration and suppression of apoptosis (Vigneron et al., 2010).

Studies have addressed the role of YAP overexpression in stimulating proliferation (Camargo et al., 2007; Dong et al., 2007; Zhao et al., 2007). Several transcription factors, including Runx2, ErbB4, p73 and TEAD in particular, have been reported to interact with YAP (Basu et al., 2003; Vassilev et al., 2001). Zhao and co-workers suggested TEAD to be one of the most potent YAP targets from a transcription-activity based screen. In addition, connective tissue growth factor (CTGF) was identified as a direct target gene resulting from the interaction between nuclear YAP and TEAD. Moreover, knock-down of CTGF was found

to abrogate YAP-stimulated cell growth reducing significantly YAP-induced colony formation (Zhao et al., 2008) .

1.4 The β-adrenergic system

1.4.1 Physiological and pathophysiological response

Adrenergic receptors, also called adrenoreceptors, are a family within the broad category of G-protein coupled receptors (GPCRs). This family of GPCRs is a target of norepinephrine, also known as noradrenaline, and epinephrine, also called adrenaline, two well-known catecholamines. By definition, a catecholamine is an organic compound that has a catechol group and a side-chain amine. In the human body, the most abundant catecholamines are epinephrine, norepinephrine and dopamine. The binding of a catecholamine to the receptor will stimulate the sympathetic nervous system; various stimulant drugs are catecholamine analogues (Rang, 2003). Adrenergic receptors are subdivided into two families, α and β, which in turn have several subtypes. α1 receptors couple to Gq alpha subunit that activates phospholipase C (PLC) which in turn hydrolyzes PIP_2, phosphatidylinositol 4,5-bisphosphate, to diacyl glycerol (DAG) and inositol triphosphate (IP_3); Activation of this results in increased cytoplasmic Ca^{2+}. DAG is a second messenger that activates PKC (Figure 1.19). Conversely, α2 couples to Gi alpha subunit causing a decrease in cyclic adenosine monophosphate, cAMP (Figure 1.19). β receptors couple to Gs alpha subunit, inducing an increase in intracellular cAMP activity (Rang, 2003) (Figure 1.19).

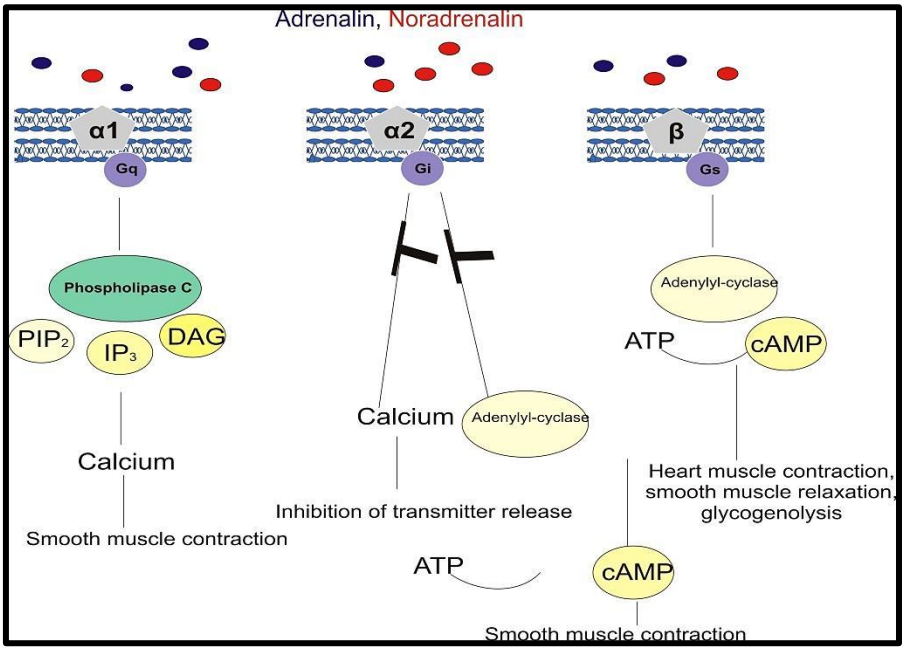


Figure 1.19 The mechanism of action of the adrenergic receptors.

1.4.2 β-adrenergic receptors

β-adrenoreceptors are members of the GPCR with seven trans-membrane spanning regions and can be subdivided into three families: β_1, β_2, β_3 (Lands et al., 1967). As previously mentioned, activation of β-receptors by specific agonists increases cAMP levels via Gs which in turn activates cAMP-dependent PKA, leading to the phosphorylation of various membrane and/or intracellular proteins.

1.4.3 β-adrenergic agonists and antagonists

β-adrenergic agonists are a class of sympathomimetic agents that act upon β-adrenoreceptors, mimicking the actions of adrenaline and noradrenaline signaling. Examples of β_1-agonists include isoproterenol and dobutamine (Rang, 2003).

β-antagonists, more commonly referred as β-blockers, are a class of drugs that block the action of endogenous catecholamines epinephrine (adrenaline) and norepinephrine (noradrenaline) (Rang, 2003). Examples of commonly used β-blockers are the non-selective agent, propranolol, the β_1 selective compound, atenolol, and the β_2 selective agent, butaxamine.

1.4.4 β-adrenergic receptors in cancer

The role of β-agonists in regulating cell proliferation is still a controversial field as there is a lack of consensus in the actions that affect β-adrenoreceptors in controlling cell replication and proliferation. Some studies have linked the use of β-adrenergic antagonists, also known as β-blockers, with reduced disease progression (Botteri et al., 2013; De Giorgi et al., 2011) and other studies reported the effect of agonists in tumour regression (Carie and Sebti, 2007; Slotkin et al., 2000).

Of particular interest to this work is the β-adrenergic receptor agonist dobutamine (Figure 1.20), as a possible compound to affect the function of pancreatic cancers. In a cell-based screen of 48 drugs aiming at identifying YAP inhibitors, dobutamine was found to be the most promising compound in preventing nuclear accumulation of YAP and YAP-mediated transcriptional activation (Bao et al., 2011).

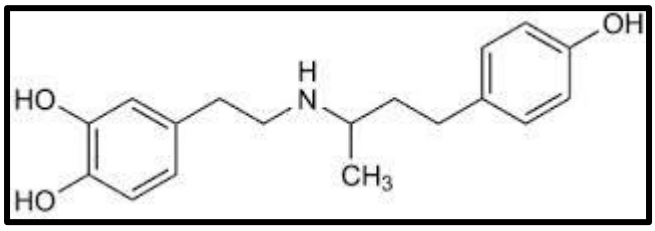


Figure 1.20 Molecular structure of dobutamine

1.5 Project aims and objectives

PDAC is one of the most lethal malignancies, having a dismal cure rate. There is an urgent need to find better ways to target therapies to or modify the behavior of PDAC. The aim of this thesis is to provide insights into functional properties of pancreatic epithelial cells as a consequence of de-differentiation and to identify ways to apply this information to improve the targeting and/or efficacy of therapies to treat PDAC.

The experimental work in this thesis is divided in three chapters. The first results chapter, Chapter 3, explores the expression and distribution of PepT1 in human pancreatic samples and investigates how PepT1 could be used to improve the selective targeting of PDAC. The following chapter, Chapter 4, examines how cell polarity, disrupted in cancer, can affect the efficacy of current chemotherapeutic strategies to treat pancreatic malignancy. Finally, in Chapter 5, it is shown how a β-adrenergic agonist might be used to improve the efficacy of a currently used chemotherapeutic strategy.

I) PepT1 as a Drug Delivery Target to Treat Chronic Pancreatitis and Pancreatic cancer.

Previous studies have reported the up-regulation of PepT1, an oligopeptide transporter system, in pancreatic cancer cells (Gonzalez et al., 1998). Hitherto, there are no studies reporting PepT1 distribution in human pancreatic biopsies from inflamed and cancerous pancreas. Past research has highlighted the interactions between drug transporters with clinically relevant anti-cancer drugs (Inui et al., 1992; Song et al., 2005; Tsume et al., 2008). The feasibility of using PepT1 to selectively deliver agents to inflamed or cancerous pancreatic cells, in the context that chronic inflammation in this organ has been identified as

a potential pre-empting or concomitant factor for oncogenesis, will be addressed. The main research objectives for this work are as it follows:

1. To analyze the expression and distribution pattern of PepT1 in pancreatic cancer cells and inflamed tissue;
2. To determine the role of PepT1 in pancreatic cancer cell proliferation;
3. To investigate the delivery of gemcitabine and ibuprofen into pancreatic cancer cells following modification to include a hydrolysis resistant thio-dipeptide, Ala(S)--Ser or Ala(S)-Asp as a means to enhance uptake through PepT1.

II) Ocln interactions with Hippo Pathway Signaling.

Several studies have suggested that the Hippo pathway signaling involved in regulating cell proliferation, apoptosis, and apical-basal polarity, could provide an important regulatory feedback loop to organize epithelial barriers (Boggiano and Fehon, 2012; Genevet and Tapon, 2011). E-cadherin localised at the AJ, has been the only element shown so far that is involved in sensing cell-cell contacts and regulates epithelial cell proliferation at high lateral cell density (Schlegelmilch et al., 2011). In addition, siRNA-mediated knock-down of E-cadherin in MDCK cells leads to disorganized AJs (Qin et al., 2005). There is a rationale to speculate that a TJ element would be similarly involved in coordinating the completion of apical-basal polarity with the suppression of cell proliferation controlled through the Hippo pathway. The research objectives for this chapter are the following:

1. To characterize potential protein-protein associations between occludin and Hippo signaling pathway elements in two pancreatic cancer cell lines with different growth characteristics;
2. To analyze the distribution of Hippo pathway elements in chronic pancreatitis and pancreatic cancer human biopsies;

3. To explore if dobutamine treatment, shown to induce YAP translocation from the nucleus to the cytoplasm, can affect occludin expression or function in two pancreatic cancer cell models;

III) Dobutamine – a potential Adjuvant Molecule in the Treatment of Pancreatic Cancer.

Certain catecholamines appear to be able to control cell division and differentiation (Slotkin et al., 2000). Dobutamine, a β-adrenergic agonist, has been reported to reduce the extent of nuclear YAP, allowing it to be degraded in the cytoplasm (Bao et al., 2011). Since YAP protein down-regulation reduces cell proliferation and clonogenicity of pancreatic cancer cells (Diep et al., 2012), the therapeutic potential of dobutamine in pancreatic cancer was analysed. The research objectives for this work are as follows:

1. To examine the cytotoxic actions of dobutamine on pancreatic cancer cell viability, proliferation and apoptosis induction;
2. To explore if dobutamine affects proliferation rates and paracellular permeability through occludin expression in pancreatic cancer cells;
3. To explore if the combined cytotoxic actions of gemcitabine and dobutamine would be beneficial as an adjuvant chemotherapeutic approach in pancreatic cancer;
4. To investigate the potential in maximizing cytotoxicity to pancreatic cancer by combining dobutamine and a dipeptide analogue of gemcitabine as PepT1 substrates.

Chapter 1

Introduction

Chapter 2

2 Materials and Methods

2.1 Cell culture

2.1.1 Methodology

2.1.1.1 Sub-culturing procedure

All cell lines used were obtained from ATCC© apart from HPDE cells, which were a generous gift from Dr. Ming-Sound-Tsao from the University of British Columbia, Toronto. HPAFII cells (Figure 2.1, Table 2.1) were grown in Minimum Essential Medium (Fisher©). AsPc-1 cells (Figure 2.1, Table 2.1) were cultured in RPMI-1640 (Fisher©), Capan2 (Figure 2.1, Table 2.1) in McCoy's (Fisher©) and HPDE in keratinocyte serum free media supplemented with L-glutamine, recombinant epidermal growth factor and bovine pituitary extract (Fisher©). All cell media except keratinocyte serum free media was supplemented with 10 % heat inactivated fetal bovine serum (Fisher©), and 100 units/mL of Penicillin and 100 µg/mL of Streptomycin (Sigma®). Cells were maintained at 37 °C in a humidified atmosphere containing 5 % CO_2. For sub-culturing procedures, cells were very briefly washed in PBS – Phosphate buffered saline. Following this, 0.25 % trypsin/EDTA (Sigma®) was added to the flask, and left for approximately 5 minutes. Afterwards, an equal amount of warmed cell medium was added. Cells were centrifuged at 125 x g for 10 minutes. Subsequently, cell medium was removed and the cell pellet re-suspended in freshly warmed cell media and transferred into one or more culturing flasks depending on the growth requirements of the cell line. This procedure was repeated twice a week for all the cell lines at a 1:2 split ratio. Due to the extreme grade of HPDE cell line sensitivity, before starting the cell culture procedure, a free standing UV lamp (radiation: 253.7 nm; 230 V and 0.5 amps) was used for approximately 30 minutes in order to more efficiently sterilise the area under the hood.

2.1.2 Freezing and thawing procedure

For thawing purposes, vials were removed from liquid nitrogen and quickly thawed at 37 °C in a water bath; warm and complete cell media was added to the cells and centrifuged for 10 minutes at 125 x g. Pellet was then re-suspended in fresh media and placed in a T25 flask. After 2 or 3 days, depending on the cell line, cells were then transferred to a T75 at a suitable split ratio. To freeze the cells, the pellet was re-suspended in antibiotic free cryopreserving media supplemented with 40 % FBS, fetal bovine serum, and 10 % DMSO; after filtering the media, cells were re-suspended at 1 x 10^6 cells/mL into cryotubes, placed at -80 °C overnight and then stored in liquid nitrogen.

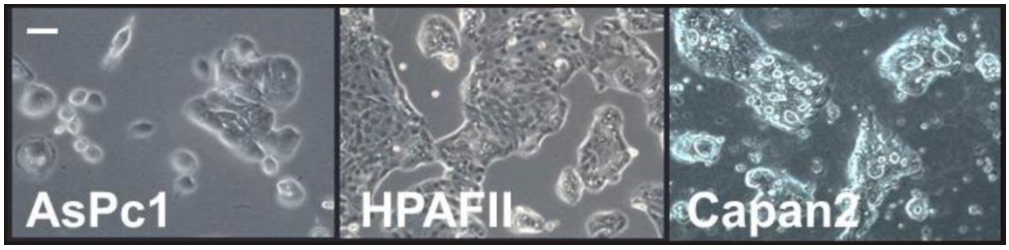


Figure 2.1 Bright field images of three cell lines studied; Scale bar, 50 µm.

Table 2.1 – Table outlining the main differences amongst the cell lines used.

Cell line	Organism	Morphology	Derivation	Tissue	Culture properties	Disease	Age (years)	Gender	Genes expressed
AsPc1	Human	Epithelial	Nude mouse xenografts initiated with cells from the ascites of a patient with pancreatic cancer	Pancreas	Adherent	DPAC	62	Female	Carcinoembryogenic antigen; human pancreas associated antigen; human pancreas specific antigen; mucin; Ras+
HPAFII	Human	Epithelial	Peritoneal ascidic fluid from an individual with primary pancreatic cancer and metastasis to the liver, diaphragm and lymph nodes.	Pancreas	Adherent	DPAC	44	Male	Blood Type A; Rh-; HLA A1, A10 (W34), B8, W22, Cw3
Capan2	Human	Epithelial	Pancreatic tumour	Pancreas	Adherent	DPAC	56	Male	Blood Type B; Rh+, The cells produce high levels of MUC-1 mucin mRNA, low levels of MUC-2 mRNA but do not express the MUC-3 gene.

2.2 Permeability measurements through the paracellular pathway

2.2.1 Theoretical background

The permeability through the paracellular pathway can be evaluated by different methods: measurement of trans-epithelial electrical resistance, TER, and the flux of fluorescent tracer solutes (FITC-dextran) (Balda et al., 1996; Blume et al., 2010).

2.2.2 TER

TER is a widely used method to determine quantitatively the tight junction dynamics and healthy state in cell culture systems (Blume et al., 2010). The traditional method for measuring TER values is to use STX2 electrodes that detect how confluent a cell monolayer is. Each electrode contains a silver/silver-chloride pellet for measuring voltage and a silver electrode for passing current. These electrodes are suitably designed to facilitate its placement into a variety of cell culture wells; one set of electrodes is placed in the basolateral compartment and the other set at the apical side. TER measurement is based on Ohm's law. It is determined by the application of a direct current with a defined voltage applied to two electrodes, one on each side of the cell layer (apical and basolateral compartments respectively). The generated current is measured, leading to the generation of ohmic resistance. Higher resistances between the electrodes will produce a higher level of confluence as fewer ions can flow in between them.

2.2.3 Fluorescent tracer solutes

Paracellular diffusion of hydrophilic tracers can be measured through the use of fluorescently labeled tracers such as dextrans. Dextran is a high molecular weight polymer of glucose and, when labelled with fluorescent dyes, allows the study of paracellular barrier properties. Fluorescently labelled dextrans, 4 kD FITC and 70 kD Rhodamine dextran are the tracers most commonly used to assess the size-selectivity of tight junction paracellular permeability.

2.3 Methodology

2.3.1 Cell seeding

HPAFII cells were seeded onto the apical chamber, a polyester filter with 0.4-μm pore size, 12 mm Transwell™ filters (Corning®) at a cell density of 7 x 10^4 per well; Cells were left in complete media (MEM) at the apical chamber, typically for two weeks to reach confluence. Media from the two compartments was removed on a daily basis to enable healthy growth. For background reading, a well was left only with media without cells.

2.3.2 TER measurements

Pre-warmed media was placed at the apical chamber and incubated at 37 °C for 30 minutes to equilibrate. The electrodes were sterilized using 70 % ethanol and placed in PBS in order to eliminate ethanol traces and calibrate the equipment. For apical-basal polarity studies, TER was determined with a pair of fixed current passing voltage measuring electrodes (World Precision Instruments©, UK) as the schematic in Figure 2.2 shows. TER values were

obtained by subtracting the contribution of the filter and bathing solution and multiplied by the surface area of the well (1.12 cm^2). Three measurements were taken per well and averaged. Final resistance values were calculated according to the formula shown below.

$$R_{true\ tissue} = R_{Total} - R_{blank}$$

$$TER = (R_{total} - R_{blank})\ \pi r^2 = ohms.cm^2$$

R_{total} – Resistance measured;

R_{blank} – resistance of the control filters

r –radius of the filters

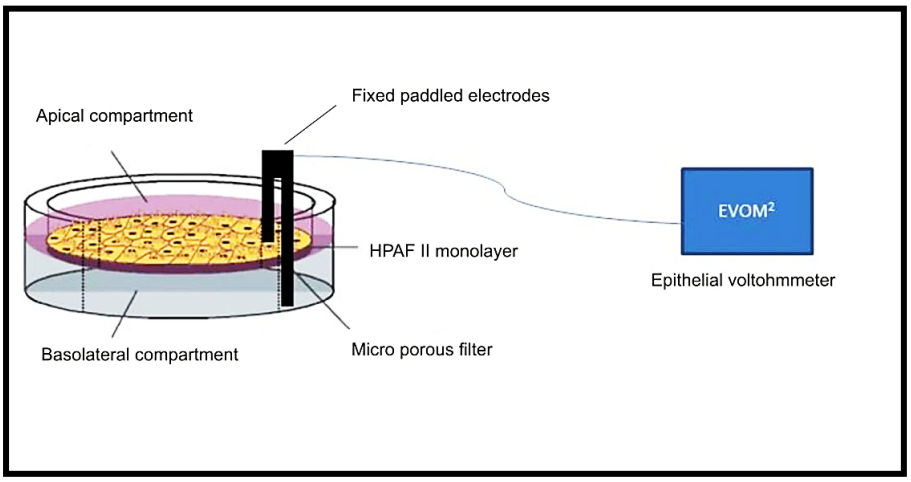


Figure 2.2 Diagram showing the measurement of TER in HPAF II cell cultures.

2.3.3 FITC Dextran permeability

Cells were plated in 12-well tissue culture inserts (as described in section 2.3.1). Culture media was replaced by fresh MEM (Fisher©) without phenol red (1 mL was added to the outer chamber and 250 µl to the inner one). Cultures were then left to equilibrate in the tissue culture incubator for at least 30 minutes. 50 µL of FITC Dextran (Sigma®) was added to the inner chamber bringing the final volume to 300 µL, so that the final concentration was 1 mg/mL. The cultures were then incubated at 37 °C for 4 h. Filters were removed and the diffused tracer measured by the FLUOstar OPTIMA (FITC-DEXTRAN: Exc: 485 nm and emission: 544 nm).

2.4 Protein extraction and subcellular fractionation

2.4.1 Methodology

2.4.1.1 Isolation of whole cell lysates

To collect cytoplasmic fractions, growth media was removed and cells were washed twice in ice-cold PBS prior to the addition of lysis buffer (50 mM Tris-HCL, pH 7.5, 150 mM NaCl and 1% NP-40) containing phosphatase and protease inhibitors. After incubation on ice for five minutes, the samples were centrifuged (8000 x g, 10 min, 4°C) to pellet the cell debris and the supernatant was carefully removed and placed in another pre-cooled Eppendorf tube. Protein concentration was measured through NanoDrop Spectrophotometer 2000 (Thermo Scientific) according to the protocol described in section 2.5.

2.4.1.2 Isolation of membrane fractions

For membrane isolation, cells were washed with PBS containing Ca^{2+} and Mg^{2+} and lifted in 50 mM Tris-HCl, pH 7.5 containing protease and phosphatase inhibitors while on ice. Collected cells were disrupted by freeze-thaw cycles prior to centrifugation (1000 x g, 10 min, and 4 ^{0}C). Membrane pellets were transferred to a Beckman ultracentrifugation tube (Beckman®) and were isolated at 100,000 x g for 1 h at 4 ^{0}C. Pellets were then washed twice in PBS containing Ca^{2+} and Mg^{2+} and then suspended in lysis buffer containing 0.1 % Triton-X-100 by leaving it on ice for up to 2 h. Protein concentration was measured through NanoDrop Spectrophotometer 2000 according to the protocol described in section 2.5.

2.4.1.3 Isolation of nuclear fractions

For nuclear fraction isolation, growth media was removed and cells were washed twice in ice-cold PBS prior to the addition of lysis buffer (50 mM Tris-HCL, pH 7.5, 150 mM NaCl and 1% NP-40) containing phosphatase and protease inhibitors. After incubation on ice for five minutes, the samples were centrifuged (8000 x g, 3 min, 4°C) to pellet the cell debris and the supernatant was carefully removed and placed in another pre-cooled Eppendorf tube. Supernatant was then centrifuged again in order to pellet the nuclear fraction. Protein concentration was measured through NanoDrop 2000 according to the protocol described in 2.5.

2.5 Protein concentration

2.5.1 Theoretical background

Quantifying protein concentration through absorption spectrophotometry is a fast, convenient and reliable technique, in which the protein can be fully recovered at the end of the quantification method. This technique is based on the fact that proteins in solution absorb UV light between 200 nm and 280 nm. When light of a specific wavelength passes through a sample, some of this light is absorbed, decreasing the amount of light that passes through the sample - transmitted light. The difference between the original and the transmitted light is called absorbance.

The determination of protein concentration through absorbance measurement is based on Beer's law, which states that molar absorptivity is constant and the absorbance is

proportional to concentration for a given substance dissolved in a given solute and measured at a given wavelength. Beer´s law can be defined as follows:

$$A= \varepsilon * b * C$$

A = absorbance; ε = wavelength-dependent molar absorptivity coefficient m^2/mol; b = path length in centimeters; c = analyte concentration in moles/liter.

Solving the expression of Beer's law for concentration yields the following equation:

$$C = A / \varepsilon b$$

Unless the ε value is known for a specific protein, this method represents merely a rough estimation of protein concentration.

2.5.2 Methodology

To ensure an equal sample loading, protein concentration was determined through Nanodrop Spectrophotometer 2000 which has a detection limit of 2 ng/µl. Samples were blanked first and the sample type option chosen was the default general reference setting in which 1 Abs = 1 mg/mL. A sample of 1.5 µL was loaded onto the lower measurement pedestal and the sampling arm lowered. Absorbance measurements were taken and spectral image was reviewed to assess sample quality.

2.6 Immunoblotting and immunoprecipitation

2.6.1 Theoretical background

2.6.1.1 Immunoblotting

Immunoblotting, also known as Western Blot, is a widely used technique for the detection of protein in a cell extract. It is based on gel electrophoresis, whereby denatured proteins are separated by the length of polypeptide. The proteins are then transferred to a PVDF or nitrocellulose membrane where they are stained through specific antibodies targeting a protein of interest. Following incubation, the membrane is exposed to a secondary antibody tagged to a HRP (horseradish peroxidase) enzyme; through chemiluminescent agents that catalyse the enzymatic action of HRP, light is emitted and then detected on a photosensitive film.

2.6.1.2 Immunoprecipitation

Immunoprecipitation is a technique that involves the precipitation of a protein antigen out of a cell lysate through a specific antibody, recognizing the protein of interest. This technique requires the antibody to be coupled to a substrate, such as agarose beads.

2.6.2 Methodology

2.6.2.1 Immunoblotting

Cells were lysed and protein quantified as described in sections 2.4 and 2.5; each sample was made up approximately to 25 µg of protein in LDS buffer (200 µL of 10x sample

reducing agent – Invitrogen™, 500 µL 4x LDS buffer - Invitrogen™, and 400 µL of ddH_2O) was added per sample and the lysates incubated for 10 minutes at 95 °C, to allow for protein denaturation; subsequently, samples were centrifuged at 200 x g and the protein extract was then separated in a 12 % acrylamide gel (250 V, 45 min) (Table 2.1 and Table 2.2). After, the gel was transferred (XCellTM Blot module; Invitrogen) onto a PVDF membrane (Thermo Scientific), previously activated by methanol, using transfer buffer (48 mM Tris-Base, 39 mM glycine, 20% methanol) and a voltage of 30 V for 1.5 h was applied. After the transfer was complete, membranes were Ponceau stained (Sigma®) to confirm the presence of transferred proteins; staining was then washed away with TBS-T (Tris buffer saline - 10 mM Tris- HCl, pH 7.5, 150 mM NaCl and 0.1% Tween 20) and then the PVDF membrane was blocked in 5 % dried non-fat milk dissolved in TBS-T overnight at 4 °C. After three washes in TBS-T at RT, appropriate secondary-HRP antibodies (GE Healthcare) were applied generally at a dilution of 1:1000 for 1 h at RT in TBS-T containing 1 % milk. Blots were washed in TBS-T with HRP activity being detected by ECL (GE Healthcare©). The film was then scanned in order to carry out densitometry measurements through the software ImageJ.

2.6.2.2 Immunoprecipitation

Cells were lysed in RIPA buffer for 10 minutes at 4°C and the cell lysate centrifuged (10,000 x g, 10 min, 4^0C). Lysates containing 500 µg of protein were incubated with a pull-down antibody overnight at 4°C. A/G beads™ (Santa Cruz Biotech) were added and the mixture was left in rotation for 2 h at 4 °C. Beads were washed three times in RIPA buffer by centrifugation at 4000 x g, 1 minute, RT. Following this, proteins were separated through gel electrophoresis and transferred to a PVDF membrane as described in section 2.6.2.1.

2.6.2.3 Membrane stripping

The membrane was placed in 1 x stripping buffer (100 mM 2-mercaptoethanol, 2 % SDS, sodium dodecyl sulfate, 62.5 mM Tris-HCl, pH 6.7) on a rocking platform in a water bath at 60 °C during 30 minutes for immunoblotting and for 1 h for immunoprecipitation blots; the membrane was then transferred to a TBS-T solution and washed three times (10 minutes per wash); After being blocked again, the membrane was incubated with the primary antibody of interest.

2.6.3 12% Polyacrylamide gel recipes

Resolving gel:

Table 2.2 Table showing the volume of each reagent used to make the resolving gel.

Reagent	Volume (mL)
MQ water	24
40 % Acrylamide	16.8
1.5 M Tris pH 8.8	14
10 % SDS	0.560
10 % APS	0.560
TEMED	0.056

Pour resolving gel into cassette and overlay with Butanol; leave for 45 minutes to polymerize.

Stacking gel:

Table 2.3 Table showing the volume of each reagent used to make the stacking gel.

Reagent	Volume (mL)
MQ water	11.6
40 % Acrylamide	3
1.5 M Tris pH 8.8	5
10 % SDS	0.200
10 % APS	0.200
TEMED	0.020

Pour stacking gel over resolving gel and insert combs.

2.7 Cell proliferation assay

2.7.1 Theoretical background

Several assays to determine cell proliferation are widely used, each of which with its own limitations. Nucleic acid content is a reliable indicator of cell number since DNA and RNA levels are tightly regulated at a cellular level and are generally independent of changes in cellular metabolism. In culture, the levels of DNA and RNA vary substantially in a single cell, however the net nucleic acid content per an individual cell remains constant over time as the culture grows (Jones et al., 2001). Thus, an assay quantifying the nucleic acid content should reflect the cell numbers accurately. The CyQUANT® (Life Technologies) assay is a rapid and sensitive method for determining the density of cells in culture. It uses a green fluorescent cyanine dye characterized by very low intrinsic fluorescence and large fluorescence enhancement once it binds to nucleic acids (Jones et al., 2001).

2.7.2 Methodology

2.7.2.1 Cell seeding and treatment

Cells were plated in white clear bottom 96-well plates (Fisher©), and allowed to attach overnight. Test compounds were added in optimal growth media and incubated for a specific time interval.

2.7.2.2 Reagent reconstitution

2.2 mL of 5x HBSS (component C) was added to 8.8 mL distilled H_2O and 22 µL of CyQuant™ dye reagent (component A) was mixed with 11 mL of 1x HBSS buffer.

2.7.2.3 Cell proliferation assay

For cell proliferation assays, treatment media was removed and 100 µL of 1 x dye binding solution (CyQuant®) was added. After a 30 minute incubation, fluorescent intensity was measured at 22 ^{0}C using a microplate reader (FLUOstar Omega; BMG Labtech) at 485/530nm excitation/emission wavelengths.

2.8 Cell viability assay

2.8.1 Theoretical background

Cell viability is an important determinant in the ability of cells or tissue to recover their functional properties after being exposed to an insult, either chemical or physical. Viability assays can be classified into 4 different categories: cytolysis or membrane leakage assays such as propidium iodide (PI) or trypan blue, mitochondrial activity or caspase assays such as formazan (MTT/ XTT), functional assays to assess motility and finally genomic and proteomic assays such as DNA microarrays. The cell viability test used for this work was formazan, hence a special focus will be placed on this specific assay. It is based on the capacity of NAD(P)H-dependent cellular oxidoreductase enzymes to reduce MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) to its insoluble formazan in living cells. Therefore reduction of MTS is directly proportional to cellular metabolic activity due to elevated NAD(P)H flux (Berridge et al., 2005). The MTS assay is a one-step MTT assay and has the convenience of adding the reagent straight to the cell culture system without extra steps required for the MTT assay.

2.8.2 Methodology

After determining the ideal seed density for each cell line (data not shown), cells diluted in culture medium were plated in 96-well plates, with white clear bottom plates, and allowed to attach for 4 h. 5 µl of each stock solution for a specific compound was added to 245 µL of cell culture media and mixed well, and 50 µL of the resulting mixture were added to each well. Each concentration was repeated four times and control cells received an equal amount of cell media alone or supplemented with 1 % DMSO. 50 µL of the cell suspension plus 50 µL of the test compound diluted in culture medium, were added to each well (100 µL per well). Plates were incubated for 24 h or 48 h at 37 °C in a humidified atmosphere, 5 % CO_2; 20 µL of MTS reagent (Promega©) was then added per well. Plates were returned to the incubator for approximately 3 h and absorbance readings were taken at OD 490 nm.

2.9 Immunostaining and Confocal Microscopy

2.9.1 Theoretical background

Confocal microscopy is an advanced microscopy technique that was patented in 1957 by Marvin Minsky whose purpose was to overcome the limitations of the conventional fluorescence microscope (Minsky, 1988). This microscopy technique allows for the production of exceptional quality and resolution images. It also enables the collection of different slices of the same specimen, allowing for the creation of a 3-D representation of the sample. The term “confocal” refers to the arrangement of two lenses to focus on the same point. There are many aspects that make the confocal microscope a much more versatile instrument than the conventional one. The major optical difference between the two is the presence of confocal pinholes that allow only light from the plane of focus to reach the

detector. In the conventional light microscope, two dimensional images of the specimen are formed in X and Y planes, generally parallel to the plane of sectioning (Figure 2.3). The same principle applies to confocal microscopy, however the microscope optics only allow the light from the plane of focus to reach the detector. The use of suitably positioned pinholes allows for the removal of the “out of focus”. The main feature of confocal microscopy that poses a major advantage is the fact that only one point in the sample is illuminated at a time, allowing for the collection of a series of images (a stack) that constitutes a high resolution 3D imaging and the surface profiling of a specimen. Conversely, in conventional microscopy, the quality of the image, the resolution and magnification rely upon the quality of objective lens used.

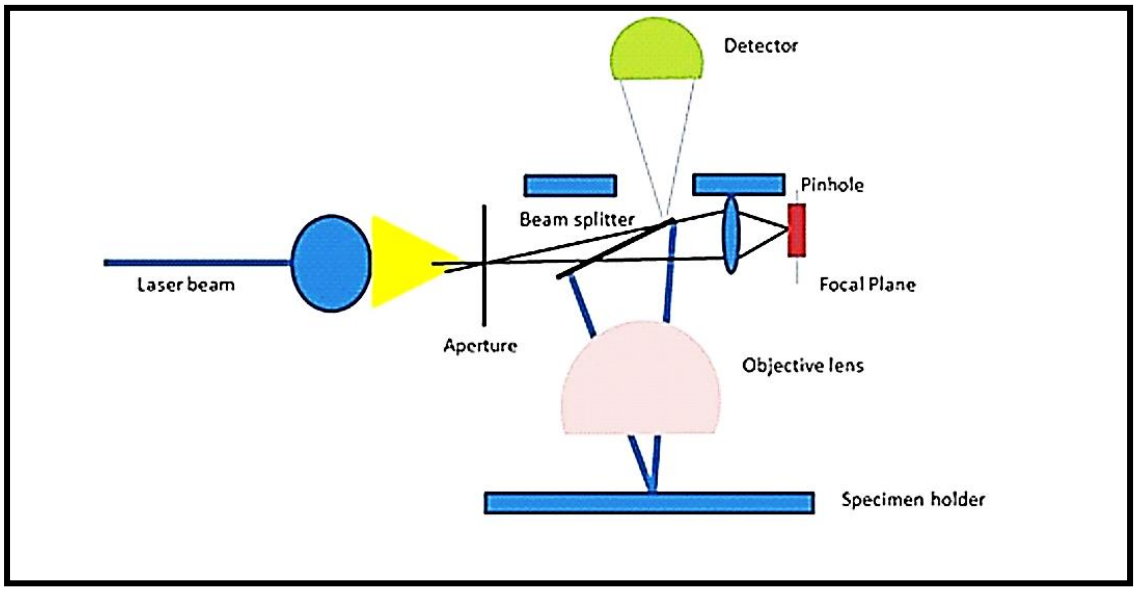


Figure 2.3 Diagram representing the main principle of the confocal microscope.

The term fluorescence refers to the emission of light of a specific wavelength which is absorbed by the fluorophores at a high-energy state, causing them to emit light of longer wavelength in a lower energy state. The emitted light is of a different color than the absorbed light (Figure 2.4).

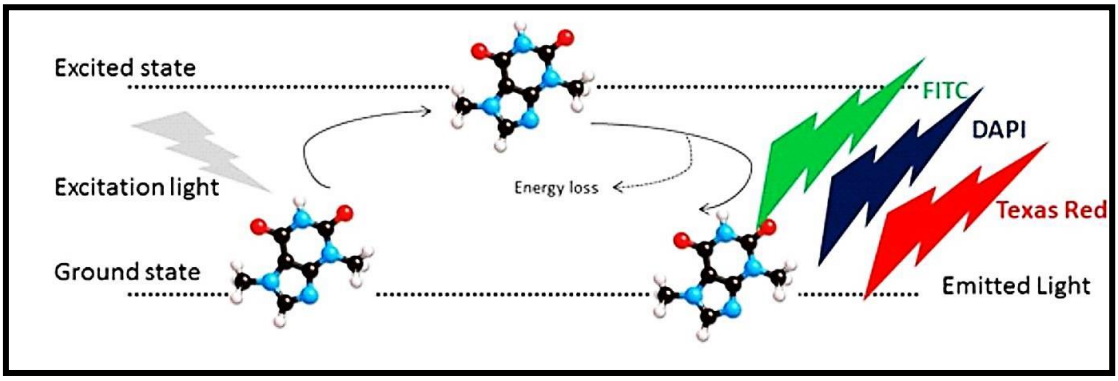


Figure 2.4 Diagram showing the principle of fluorescence imaging and the emitted light colors of the three main dyes.

In biology, fluorescence microscopy is a powerful tool that allows examining the distribution and localisation of a specific protein within a sample. There is a wide range of techniques that allow for the fluorescent analysis of biological samples. Some fluorescent stains are specifically designed to bind to an intrinsic target within the sample. An example of this biological fluorescent stains is DAPI (4',6-diamidino-2-phenylindole) which binds strongly to A-T rich regions in DNA specifically labelling the nucleus.

Immunofluorescence is another technique which uses a highly specific fluorescently labelled antibody to its respective antigen, labelling the proteins or other molecules of interest at the membrane or inside the cell.

2.9.2 Methodology

Cells were grown on coverslips for the required exposure time. For membrane protein detection, cells were washed three times with 10 mM HEPES buffer containing 0.9 % $CaCl_2$, and then fixed with 4 % PFA, paraformaldehyde, for 20 min. For cytosolic proteins, cells were washed with PBS and then fixed with 4 % PFA. Cells were simultaneously blocked and permeabilised for 30 minutes at 4 °C in blocking buffer (PBS containing 2 % BSA - bovine

serum albumin and 0.5 % Triton X-100). Cells were then washed, either with 10 mM HEPES buffer containing 0.9 % $CaCl_2$ or PBS and incubated for 2 h at 4 °C with a primary antibody diluted 1:100 in blocking buffer and then a secondary antibody diluted 1:400 in blocking buffer for 30 minutes. 1 µg/mL solution of DAPI was applied on coverslips for 20 minutes and cells were mounted on MOWIOL 4-88 (Sigma®), which has the same refractive index as immersion oil. Slides were then left to dry overnight and the following day were examined through Zeiss Confocal Laser Scanning Microscope using four laser wavelengths: Blue diode laser: 405 nm; Argon 488 nm, HeNe 543 nm and HeNe 633 nm (Table 2.3). Controls for immunostaining technique are in Figure 8.5, Chapter 8.

Table 2.4 List of the used dyes.

Name	Type	*Laser*	Emission wavelength	Application
DAPI	Nuclear dye	Blue Diode laser	405 nm	Nuclear labelling
FITC	Fluorochrome	Argon	488 nm	Most widely used green dye
Texas Red	Fluorochrome	HeNe	595 – 620 nm	Texas Red dye

Abbreviations – DAPI - 4', 6-diamidino-2-phenylindole; FITC - Fluorescein isothiocyanate; HeNe - helium–neon laser;

2.10 Immunohistochemistry – Paraffin staining

2.10.1 Theoretical background

Immunohistochemistry, or IHC, is a method for the detection and localisation of proteins in intact tissue sections. This technique is particularly useful for assessing the progression and treatment of cancer and is based on the specificity of antibodies to target the protein of interest in the tissue section. The interaction between the antigen and antibody can then be

visualized by two different reactions, chromogenic and fluorescence. In chromogenic detection, an enzyme conjugated to the antibody, typically peroxidase, is used to cleave a substrate producing color at the location of the protein of interest. Fluorescent detection involves the conjugation of a fluorophore to the antibody, which then can be visualized through fluorescence microscopy.

2.10.2 Methodology

2.10.2.1 Sample preparation – Dewaxing and rehydration

Tissue sections were dewaxed in Histoclear (Fisher©) for approximately 10 minutes and rehydrated through different grades of ethanol (100%, 90%, 70% and 50%) at 10 minutes each and then washed in PBS with gentle shaking for 5 minutes.

2.10.2.2 Sample preparation – Antigen retrieval

HIAR (heat induced antigen retrieval) was performed with 10 mM of citrate buffer pH 6.0. The buffer was heated up in a pressure cooker (Morphy Richards®) for approximately 10 minutes, and the de-waxed slides were added to the citrate buffer and heated in a pressure cooker for about 15 minutes (Shi et al., 1993). Further to this, sections were heated again during 5 minutes at a lower temperature. Tissue sections were allowed to reach RT for about 20 minutes.

2.10.2.3 Antigen detection

Tissue sections were blocked for 30 minutes (HRP detection using Rabbit Elite ABC Kit - Vector Labs©). The primary antibody was diluted in 0.1 % BSA in PBS at a 1:100 dilution, and incubated overnight at 4 °C in a humid chamber. The control antibody used was either Rabbit (DA1E, Cell Signaling©) or Mouse (G3A1, Cell Signaling©) depending on the immunoglobulin type as the test antibody. After washing the slides in PBS, the biotinylated secondary antibody was added for 30 minutes. Following a PBS wash, sections were exposed to 30 % (w/w) Hydrogen Peroxide (Sigma©) in order to quench the endogenous peroxidase activity. Following a further wash in buffer, the slides were incubated again at RT in Avidin:Biotin enzyme complex (Vecstatin Elite ABC kit, Vector laboratories©) for 30 minutes. The slides were washed and incubated in 3, 3' – diaminobenzidine (DAB, Vector Laboratories©) according to the manufacturer's instructions and then washed immediately with tap water for 5 minutes. Tissue was stained with Meyer´s Haematoxylin (Sigma©) for 2 minutes and rinsed under the tap water. Finally, the pancreatic biopsies were rehydrated through different grades of alcohol (50 %, 70 %, 90 %, 100 %) at 10 minutes each and dewaxed in Histoclear for 10 minutes. Further to a final wash in PBS, tissues were mounted in DePeX glue (Sigma©) and allowed to dry overnight.

2.10.2.4 Image analysis

Tissue sections were imaged using a DMI 3000B Microscope (Leica Microsystems©), and the software used for image analysis was Adobe Photoshop, version 3.0 (Adobe Systems, Mountain View©, CA).

2.10.2.5 Patient samples and informed consent

Tissue samples were a kind gift from Dr. Mert Erkan, Technical University of Munich, Germany; these were collected from patients diagnosed with PDAC following pancreatic resection. Normal pancreatic tissue samples were obtained through an organ donor procurement program whenever there was no suitable recipient for pancreas transplantation. Tissues were fixed in 5 % PFA solution for 12-24 h and then embedded in paraffin for histological analysis (Erkan et al., 2005). All patients were informed, written consent was obtained, and human protocols were approved by the Ethics Committees of the University of Heidelberg (Germany) and the Technical University of Munich (Germany), as referred in Erkan, *et al*. (Erkan et al., 2005).

2.11 Gene knock-down

2.11.1 Gene knock-down through RNA interference

Gene knock-down is an experimental technique which aims to reduce the expression of one or more genes. This can be achieved through two different ways, either through genetic modification or by treatment with a reagent such as short DNA or RNA.

RNAi (RNA interference) was discovered for the first time in *Caenorhabditis elegans* as a gene silencing mechanism in response to an injection of dsRNA (double-stranded RNA) (Fire et al., 1998; Hannon, 2002). RNAi interference can be delivered through two different methods: shRNA (small hairpin RNA or short hairpin RNA) and siRNA (small interfering RNA).

2.11.2 Stable gene knock-down through lentiviral particles

Stable RNAi cell lines, also known as knock-down stable cell lines, are cells that have integrated in their genome shRNA or a plasmid DNA to permanently silence the expression of a target gene through mRNA degradation (Paddison et al., 2002). shRNA is an RNA sequence that makes a tight hairpin structure which can be used to silence gene expression. The introduction of shRNA into mammalian cells is typically achieved through the use of viral or bacterial vectors that allow for a stable integration of shRNA and long-term knock-down of the targeted gene (Moore et al., 2010). These cell line models provide very valuable information on gene functions, long-term silencing effects and pathway signaling; however the generation of these biological systems is a very complex and lengthy process. Additional biological mechanisms can be used to regulate the gene silencing machinery such as the co-transfection of marker genes expressing antibiotic resistance in the presence of an antibiotic selecting agent. The cells with the ability to proliferate and divide are then isolated and characterized (for example Puromycin-induced stable cell lines).

A variety of viral vectors can be used to successfully obtain shRNA expression in cells such as adenoviruses, associated adenoviruses and lentiviruses (Lombardo et al., 2008). The lentiviral system is based on the HIV (human immunodeficiency virus) and has the unique ability to infect non-dividing cells, which makes this system one of the most efficient methods of a gene delivery vector. Structurally, this system uses three plasmids, pREV, pGAG/Pol and pVSVG, which are common to all retrovirus and a final plasmid encoding for the vector to be inserted into the host cell (Dull et al., 1998). pREV and pGAG/Pol encodes for viral proteins required for efficient viral packaging, whereas pVSVG encodes for a protein in the viral envelope conferring the viral particle the ability to transduce a broad range of cell types (Tiscornia et al., 2006). These plasmids are chemically transfected into a host cell that in

turn will release viral particles into the cell culture medium, which can then be used to infect the target cells (Figure 2.5). Once the vector has incorporated into the host cell genome, the shRNA is transcribed in the nucleus through RNA polymerase III. The transcript is then integrated into the host cell genome and it mimics the large RNA precursors designated by pri-microRNA, which later is processed by a class 2 RNase III enzyme called Drosha. The resulting product is exported from the nucleus by a family that mediates the transport of proteins and other cargo between the nuclear and cytoplasmic compartments called Exportin 5; the complex is then processed by Dicer (an enzyme with endonuclease activity) and loaded into a multi-protein complex that incorporates one strand of siRNA or microRNA (miRNA) called RNA-induced silencing complex (RISC); The sense strand is degraded whereas the antisense directs the RISC complex to mRNA that has a complementary sequence. In case of perfect complementarity, RISC cleaves the mRNA (cleavage mechanism). In the situation of imperfect complementarity, RISC represses the translation of mRNA (bypass mechanism). In both cases, the shRNA leads to gene silencing (Hannon, 2002).

2.11.3 Transient gene knock-down

The simplest method for RNAi is the cytosolic delivery of siRNA oligonucleotides, however this technique is limited to cells capable of transfection and is primarily used during transient *in vitro* studies. siRNA is a class of double stranded RNA molecules, 20-25 base pairs in length with phosphorylated 5` ends and hydroxylated 3` ends; siRNA is able to interfere transiently with the expression of specific genes with complementary nucleotide sequences (Bernstein et al., 2001). The success of siRNA in gene knock-down largely relies upon its binding ability to the RISC complex. Following this, the complex gets cleaved through endonucleases. The non-cleaved anti-sense strand-RISC complex can then bind to target

mRNAs to initiate transcriptional silencing (Bernstein et al., 2001). siRNA is an important tool for validating gene function and drug targeting; any gene can be knocked down by a synthetic siRNA with a complementary sequence (Bernstein et al., 2001).

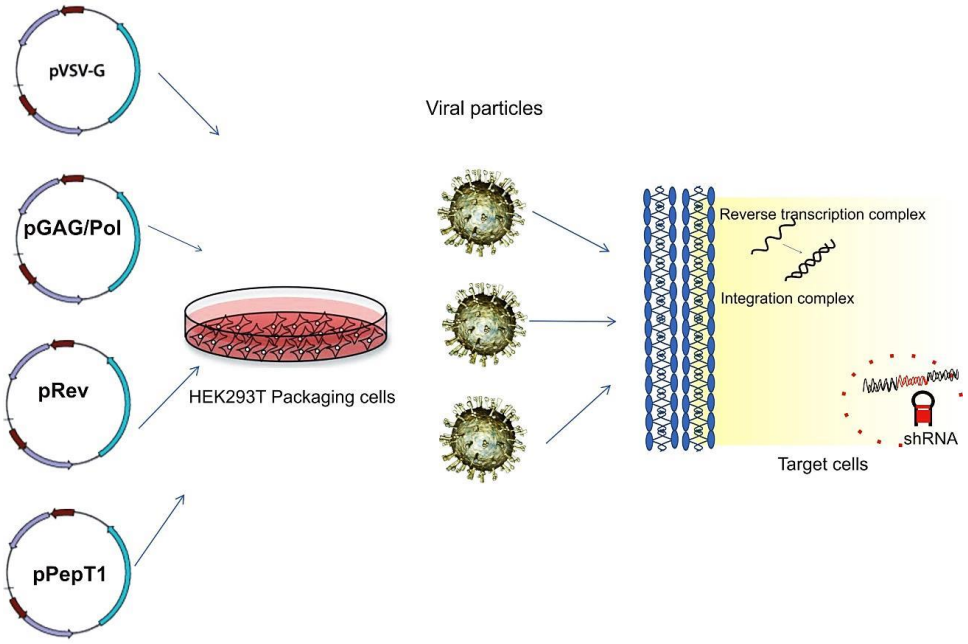


Figure 2.5 Diagram illustrating the process of lentiviral particles production.

2.11.4 Methodology: Stable PepT1 knock-down

2.11.4.1 Lentiviral generation: bacterial transformation and plasmid preparation

In order to isolate shRNA from *E. coli* (Sigma Mission®), agar plates were prepared and a final concentration of 100 µg / mL of ampicillin was added per plate. Simultaneously LB media solution was made and ampicillin was added at a final concentration of 100 µg / mL. After dried, the agar plate was streaked from a glycerol stock and left incubating at 37 °C overnight. On the following day, a colony was picked and a start culture inoculated (2-5 mL LB media containing the selective antibiotic) and left at 37 °C in an orbital shaker for approximately 8 h; after this an overnight culture was prepared (50 µL were taken to 50 mL of LB media). The following day, bacterial cells were harvested by centrifugation at 6000 x g

for 15 minutes at 4 °C to stop any further bacterial growth; Plasmids were then isolated from the bacterial culture using midi prep kits (Qiagen, Manchester, UK) following the manufacturer's instructions.

2.11.4.2 Lentiviral generation: preparation of lentiviral particles

HEK293T were plated and grown overnight to approximately 60-70 % confluence in a T75 tissue culture flask; 4 h prior to transfection the media was replaced by 10 mL of DMEM supplemented with 10 % FBS and 25 mM HEPES (pH 7.4). 7.7 µg of pREV, 7.71 µg pGAG/Pol, 3 µg pVSVG and 12.84 µg of shPepT1 were added to 62.52 µL of ddH2O to a final DNA concentration of 0.5 µg/µL. Plasmids were added to 90 µL of Mirus LT1 transfection reagent (Cambridge Bioscience, Cambridge, UK) and then to 750 µL of DMEM and left incubating for 45 minutes; subsequently the mixture was added to HEK293T cells and left for 48 h at 37 °C in a 5 % CO_2 incubator. After this, the supernatant was collected and centrifuged at 1000 rpm for 5 minutes to remove any cellular debris and divided into aliquot volumes and finally stored at -80 °C until use.

2.11.4.3 Lentiviral infection and cell selection

AsPc-1 cells were grown to 25-30 % confluence prior to infection with viral particles; cell culture medium was removed and replaced with fresh RPMI supplemented with 1 mL aliquot of viral particles. Following this, fresh cell culture medium containing 10 µg/mL of puromycin was added to select infected cells. After a 72 h period of selection, cells were harvested and lysed; PepT1 expression was assessed through Western Blotting.

2.11.5 Methodology: Transient knock-down through siRNA

A pool of 4 individual siRNA (Accell Human OCLN, siRNA-SMARTpool, Fisher© – Table 2.4) targeting different sequences in the Ocln gene was used. For proliferation assays HPAFII and AsPc-1 were grown on a 96 well plate until approximately 70% confluent. A transfection mixture of 1 µL of HiPerfect, 0.02 µL of siRNA (20 µM stock) or 0.6 µL of scrambled siRNA was added to 7.5 µL of serum- and antibiotic-free media and rocked for 15 minutes at RT. 172 µL of fresh media with or without testing compounds was added to each well in drop-wise fashion and cells were then incubated at 37 °C in 5 % CO_2 for the required exposure time. Knock-down was then confirmed through Western Blot as described in section 2.6.

Table 2.5 Sequences of siRNA used to knock-down human Ocln; Sequences were prepared as SMARTpool and used for transfections.

Species	Target	Sequence
Human	Ocln	GCAUUAACUAGCUGGAUGU
Human		CCCGUUUGGAUAAAGAAUU
Human		GUCAGAGGUUUAGAUUAGA
Human		CUCUUCAACUGUUAGAUUC

2.12 Cell death: apoptosis and necrosis

Apoptosis and necrosis are two morphologically and biochemically different forms of eukaryotic cell death (Stehbens, 2000). The term apoptosis was introduced in 1964 by Lockshin and Williams and it was described as a programmed cell death that occurs at predictable times and in certain anatomical places during embryogenesis and development of the organism (Lockshin and Williams, 1964). Later, Kerr and coauthors described the morphological characteristics during cell death and suggested the term apoptosis (Kerr et

al., 1972). Apoptosis is characterized by a sequential set of morphological events such as cellular shrinkage with condensation of the cytoplasm, nuclear fragmentation also known as karyorrhexis, deposition of chromatin masses in the nuclear membrane and formation of apoptotic bodies containing cytoplasmic organelles and nuclear fragments (Saraste and Pulkki, 2000). In apoptosis, mitochondria appear to be normal or shrunken rather than dilated or swollen, as happens in necrosis (Krysko et al., 2008). Necrosis is characterized by swelling of endoplasmic reticulum and mitochondria followed by collapse of the plasma membrane and lysis of the cells. This type of cell death is often considered a passive process lacking underlying signaling, as necrotic cells do not follow the apoptotic signal transduction pathway that involves different receptors. Identification of necrotic cell death is rather challenging, as no surface or biochemical markers have been identified yet and only negative markers are available (Krysko et al., 2008); These include the presence of differential kinetics of cell death markers such as phosphatidylserine exposure or cell membrane permeabilisation and the absence of apoptotic features such as cytochrome c release, caspase activation and oligonucleosomal DNA fragmentation (Krysko et al., 2008). Annexin V staining has been widely accepted as an apoptotic marker (Schmid et al., 1994); this Ca^{2+} - dependent phospholipid-binding protein binds strongly to phosphatidylserine residues on the cell membrane. In a normal and living cell, these residues are located in the inner surface of the membrane thus inaccessible to annexin V, however at the early stages of apoptosis, the cell membrane becomes permeable and the phosphatidylserine residues translocate to the outside of the cell (Kroemer et al., 1995). Any cell committed to apoptosis will have exposed phosphatidylserine residues allowing annexin V binding; if the annexin V is labeled with a fluorescein molecule such as FITC, an apoptotic signal will be emitted and quantified. Another way of quantifying differentially the percentage of cells that undergo apoptosis or necrosis is through FACS (Fluorescence-activated cell sorting – Figure 2.6). This technique allows for the detection of morphological changes during apoptosis and

necrosis through their light-scattering properties; the forward scatter reflects the cell size, the sideward scatter indicates the extension of cell granularity and membrane integrity can be assessed through PI uptake. Another method for cell death detection is the quantification of nucleosomes in the cytoplasm. As previously mentioned, nuclear condensation and plasma membrane blebbing are two morphological characteristics of apoptotic cell death. Activation of endonucleases leads to DNA fragmentation and consequently release of oligonucleosomes into the cytoplasm (Golstein et al., 1991). The enrichment of mono- and oligonucleosomes in the cytoplasm occurs several hours before plasma membrane breaks down. Nucleosomes in cytoplasmic lysate can be captured onto ELISA plates coated with monoclonal antibodies that recognize histones allowing for the detection of early stage apoptosis (Salgame et al., 1997).

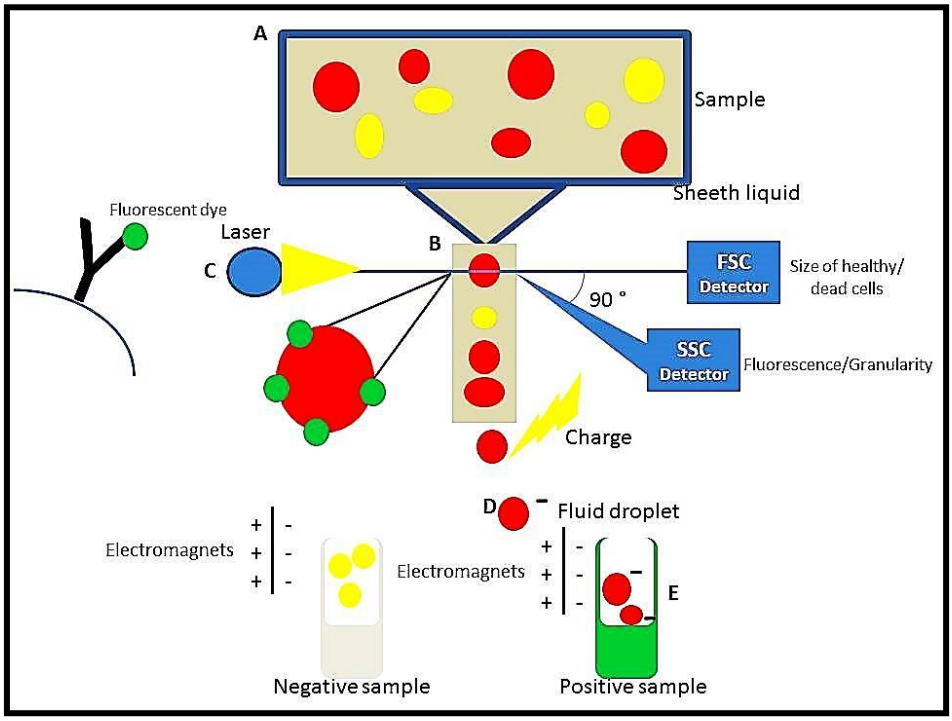


Figure 2.6 Diagram showing the principle of FACS; through light scattering and fluorescent features, this technique provides a method for sorting a heterogeneous population of cells **(A)** into two or more containers. The cell suspension flows through a stream of liquid specifically arranged to enable the separation between cells relative to their specific diameter **(B)**. The flow goes through a fluorescence measuring station in which the specific fluorescent pattern emitted by each cell is measured **(C)**. Through a vibrational mechanism the streams of cells are separated into individual droplets and an electrical charging ring is placed in the region where the stream breaks into droplets **(D)**; the opposite charge gets trapped on the droplet as it breaks from the stream. Depending on their charge, droplets fall through an electrostatic detection system that separates according to their charge **(E)**. ***Abbreviations: FSC – forward scattered light; SSC – side scattered light.***

2.12.1 Methodology

2.12.1.1 Annexin and Propidium iodide staining

For FACS, approximately 1.0 X 10^6 cells were washed in PBS and centrifuged at 200 x g for 5 minutes; cell pellet was then re-suspended in 100 µL of annexin-V-FLUOS labelling solution - 20 µL annexin-V-FLUOS labelling reagent and 20 µL Propidium iodide were diluted in 1 mL incubation buffer (Roche) and incubated for 15 minutes at 25 ^{0}C. Following

this, cells were either analysed by fluorescence microscopy, following the protocol described in section 2.9, or through FACS (for FITC staining the filter used was BP530/30: 515-545 nm and for PI was BP582/42: 561-603 nm).

2.12.1.2 Photometric enzyme immunoassay for the *in vitro* detection of cytoplasmic histone-associated DNA fragments

After inducing cell death, cells were trypsinized and centrifuged at 125 x g for 10 minutes following the protocol described in section 2.1.1.1. Supernatant was pre-diluted 1 in 10 with incubation buffer (1 x 10^4 cells/mL); 100 µL of coating solution was pipetted into each well of the plate modules and covered with adhesive cover foil. After 1 h incubation at 20 ^{0}C, coating solution was removed; 200 µL of incubation buffer was pipetted into each well and plate was incubated for 30 minutes at 20 ^{0}C; after rinsing the wells three times with washing solution, 100 µL of sample solution was sampled (sample diluted in incubation buffer 1 in 10) into each module; after a 90 minute incubation at 20 ^{0}C, 100 µL of substrate solution was pipetted into each well; plate was then incubated on a plate shaker at 250 rpm for 10 minutes until color development was sufficient for photometric analysis. Finally, absorbance readings were taken at 450 nm through FLUOstar Omega; BMG Labtech.

2.13 Statistical analysis

Data are represented as the mean of 3 independent experiments ± SEM. Statistical analysis was performed using GraphPad Prism 5 software. Significant differences between experimental and control groups were determined by one-way ANOVA followed by Dunnet's post hoc test for experiments in which three or more groups were compared. A paired t-test

was also used when comparing only two groups. A statistically difference was accepted if the p values were less than 0.05. Significance is indicated on the graphs as it follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

2.14 List of materials and reagents used

Table 2.6 List of reagents and materials.

Reagent / Material	Company / catalogue number	Application
12 well plates	Fisher© / 07-200-82	*In vitro* assays
2-mercaptoethanol	Sigma® / M3148	Membrane stripping
30 % Acrylamide/Bis	Bio-rad / 161-0154	Gel electrophoresis
30 % Hydrogen peroxyde	Sigma® / H1009	Immunohistochemistry
6 well plates	Fisher© / CLS3335-50EA	*In vitro* assays
96 well plates	Fisher© / 10212811	*In vitro* assays
A/G beads	Santa Cruz Biotech / sc-2003	Immunoprecipitation
Accell Human OCLN, siRNA-SMARTpool	Fisher© / E-187897-00-0005	Transient knock-down
Ammonium Persulphate (AMPS)	Fisher© / A/P470/46	Gel electrophoresis
Ampicilin	Sigma® / A9393	Stable knock-down
Annexin-V-FLUOS staining kit	Roche / 11858777001	In vitro apoptotic / necrotic staining
AsPc-1 Cell line	ATCC© / CRL-1682	*In vitro* cell experiments
Atenolol	Sigma® / A7655	β-adrenergic mediation
BCH	Sigma® / A7902	System L transporter inhibitor
Beckman ultracentrifugation tubes	Beckman / 362333	Cell Membrane fractionation
BSA	Sigma® / A7906	Blocking buffer
Butan-1-ol	Fisher© / 11413543	Resolving gel
Capan2 Cell line	ATCC© / HTB-80	*In vitro* cell experiments
Cell death detection ELISA	Roche / 11544675001	*In vitro* apoptotic quantification
Cell scraper 30 mm blade length	Fisher© / 10370881	Cell lysis
Centrifuge tubes conical, 15 mL	Stores / TES-136-110R	Cell culture
Chemically modified Gemcitabine	Gift from Prof. Patrick Bailey and Gayle Wilson, University of Keele	*In vitro* cell experiments
Chemically modified Ibuprofen	Gift from Prof. Patrick Bailey and Gayle Wilson, University of Keele	*In vitro* cell experiments
CyQuant™	Fisher© / 10709253	Cell proliferation assay
DAB	Vector labs Ltd / SK-4100	Immunohistochemistry
DAPI	Sigma® / 32670	Immunostaining
DePex mounting media	Fisher© / 10050080	Immunohistochemistry
DMEM	Fisher© / 51445C	Cell culture
DMSO	Sigma® / D8418	Solvent
Dobutamine Hydrochloride	Sigma® / D0676	*In vitro* cell experiments
Dymethyl sulphoxide	Fisher© / BP231-100	Solvent
ECL	GE Healthcare© / RPN2232	Western Blot
Epithelial voltmeter	World Precision Instruments©	TER measurement

Extra thick blot paper – absorbent filter paper	Bio-Rad / 170-3966	Western Blot
FACS tubes	Scientific lab. supplies / PK1000	FACS
FBS	Invitrogen / 10500064	Cell culture
FITC-Dextran 4 KDa	Sigma® / 46944	Paracellular studies
Gel cassettes 1.0 mm	Fisher© / VXNC 2010	Gel electrophoresis
Gel loading tips	Sigma® / CLS4853-400EA	Sample loading
Gemcitabine hydrochloride	Sigma® / G6423	*In vitro* cell experiments
Glass coverslips	Fisher© / MNJ-150020A	Immunostaining
GlySar	Sigma® / G3127	Competitive in vitro cell assays
Haematoxylin	Sigma® / H9627	Immunohistochemistry
Heat inactivated fetal bovine serum	Fisher© / 10082139	Cell culture
Hepes	Sigma® / H3784	Wash buffer
HiPerfect	Qiagen / 301802	Transient knock-down
Histoclear	Fisher© / HIS-010-010S	Immunohistochemistry
HPAFII Cell line	ATCC© / CRL-1997	*In vitro* cell experiments
HPDE Cell line	Gift from Dr. Ming-Sound Tsao, Toronto	*In vitro* cell experiments
Human recombinant epidermal growth factor and bovine pituitary extract	Fisher© / 37000-015	Cell culture
Hydrophobic pen	Vector / H-4000	Immunohistochemistry
Ibuprofen	Sigma® / I4883	*In vitro* cell experiments
IFN γ	Miltenyi Biotec / 130-097-068	Cell inflammation
ImmEdge Hydrophobic Barrier Pen	Vector labs Ltd / H-4000	Immunohistochemistry
Keratinocyte – SFM supplemented with L-Glutamine	Fisher© / 17005-034	Cell culture
LB media	Fisher© / BP9723-500	Stable knock-down
LDS buffer 4x	Invitrogen™ // NP0008	Western Blot
LT1	Mirus / MIR2300	Stable knock-down
McCoy's	Fisher© / 11511861	Cell culture
MEM	Fisher© / 11514426	Cell culture
MEM without phenol red	Fisher© / 51200-038	Paracellular permeability
Micro dishes high glass bottom	Thistle Scientific Ltd.	Live cell staining
Microscope slide superfrost	Fisher© / 12372098	Immunostaining
Microscope slide superfrost	Fisher© / MNJ 750-010B	Immunostaining
Milk powder	Marvel	Western blot
Mowiol 4-88	Sigma® / 81381	Mounting media
MTT	Promega® / G3582	Cell viability assay
MYR-PKCζ	Fisher© / 10717873	Cell polarity disruption
NanoDrop 2000	Thermo Scientific	Protein quantification
Nonidet P-40	Fisher® / PI85124	Cell lysis
Pancreatic Stellate Cells	Gift from Dr. Mert Erkan, Technical University of Munich	Immunohistochemistry
Pancreatic tissue biopsies	Gift from Dr. Mert Erkan, Technical University of Munich	Immunohistochemistry
PBS tablets	Sigma® / P4417	Immunohistochemistry, cell lysis, cell culture.
Penicillin/Streptomycin	Sigma® / P4333	Cell culture
PFA	Sigma® / 158127	Immunostaining
Phosphatase Inhibitors cocktail	Fisher© / 12851650	Cell lysis
Pipette Pasteur	Fisher© / 11566963	In vitro cell experiments
Ponceau staining	Sigma® / P7170	Western Blot
Pressure Cooker	Morphy Richards	Immunohistochemistry
Propranolol	Sigma® / P8688	β-adrenergic mediation
Protease Inhibitors cocktail	Fisher© / 12841640	Cell lysis
Protein ladder	Fisher® / BP3603500	Gel electrophoresis
Protein ladder 10-170 KDa	Fisher© / 11478503	Western Blot

PSC cell lysates	Gift from Dr. Mert Erkan, Technical University of Munich	Protein expression
Puromycin	Sigma© / P9620	Cell selection
PVDF membrane	Thermo Scientific / 10617354	Western Blot
Qiagen midi prep kit	Qiagen / 12143	Plasmid isolation
RIPA buffer	Fisher© / 10017003	Cell lysis
RNAse free water	Fisher© / BPE561-1	Plasmid isolation
RPMI-1640	Fisher© / 10418243	Cell culture
Sample reducing agent 10x	Invitrogen™ / NP0004	Western Blot
SDS	Fisher© / S/5200153	Gel electrophoresis
Serological pipettes 25 mL	Fisher© / 11517752	Sample measurement
siRNA - scrambled	Fisher© / 11841984	Transient protein knock-down
shRNA PepT1	Sigma Mission® / SHCLNG	Stable gene knock-down
Slide box storage for slides	Fisher© / 11730594	Slides storage
Sodium citrate	Sigma® / W302600	Immunohistochemistry
Staining dish set	Fisher© / MNK-710-010T	Immunohistochemistry
Staurosporine	Sigma® / S4400	Induction of cell apoptosis
T 25 flasks	Fisher© / 156367	Cell culture
T 75 flasks	Fisher© / TKT-130-210T	Cell culture
TEMED	Fisher© / T/P190104	Gel electrophoresis
TGF-β	Miltenyi Biotec / 130-095-067	Cell inflammation
TNFα	Miltenyi Biotec / 130-094-014	Cell inflammation
Trans-well filters with polyester membrane	Fisher© / CLS3460	Cell polarity assays
Triton X-100	Sigma© / T8787	Detergent
Trizma base (Tris)	Sigma® / T1503	Buffer
Tween 20	Fisher© / P1379	Cell lysis; immunohistochemistry
VECTASTAIN Elite ABC Kit (Rabbit IgG)	Vector labs Ltd / PK-6101	Immunohistochemistry
X Cell Blot Module	Fisher© / 10073582	Western Blot
Y-27632	Sigma© / Y0503	Cell polarity disruption

Table 2.7 Table showing the antibodies used for each technique

				Western Blotting		Immunofluorescence		Immunohistochemistry	
Target of primary antibody	**Species**	**Molecular weight**	**Catalogue no / Company / Concentration as suplied by the manufacturer**	**Blocking buffer**	**Primary/Secondary antibody dilution**	**Blocking buffer**	**Primary antibody dilution**	**Blocking buffer**	**Primary antibody dilution**
Tricellulin	Rabbit		HPA018119 / Sigma / 100 µg / mL					0.1 % BSA in 1X PBS	1 in 100
E-cadherin	Rabbit		3195 / Cell Signalling / 0.1 mg / mL			0.2 % BSA and 0.5 % Triton-X in 1X PBS	1 in 100		
CRB3	Mouse		SAB1408190 / Sigma / 0.5 mg / mL			0.2 % BSA and 0.5 % Triton-X in 1X PBS	1 in 100		
Tricellulin	Rabbit	65 kDa	700191 / Invitrogen / 0.5 mg / mL	5% Milk in TBS-T	1 in 1000				
TEAD	Rabbit	50 kDa	8526 / Cell Signalling / 0.1 mg / mL	5% Milk in TBS-T	1 in 1000	0.2 % BSA and 0.5 % Triton-X in 1X PBS	1 in 100	0.1 % BSA in 1X PBS	1 in 100
c-Yes	Mouse	62 kDa	sc-8403 / Santa Cruz / 200 µg / mL	5% Milk in TBS-T	1 in 1000			0.1 % BSA in 1X PBS	1 in 100
HDAC	Mouse	60 kDa	sc-7872 / Santa Cruz / 200 µg / mL	5% Milk in TBS-T	1 in 1000				
c-Yes	Rabbit		sc-14 / Santa Cruz / 200 µg / mL					0.1 % BSA in 1X PBS	1 in 100
PepT1	Rabbit	79 kDa	Custom made Peptide synthesis-Innovagen / 0.5 mg / mL	5% Milk in TBS-T	1 in 1000				
PepT1	Rabbit		HPA002827 / Sigma / 0.2 mg / mL					0.1 % BSA in 1X PBS	1 in 100
YAP	Rabbit		4912 / Cell Signalling / 0.1 mg/ mL			0.2 % BSA and 0.5 % Triton-X in 1X PBS	1 in 100	0.1 % BSA in 1X PBS	1 in 100
Occludin	Rabbit	65 kDa	33-1500 / Invitrogen / 0.5 mg / mL	5% BSA in TBS-T	1 in 1000	0.2 % BSA and 0.5 % Triton-X in 1X PBS	1 in 100		
Occludin	Mouse	65 kDa	71-1500 / Invitrogen / 0.25 mg / mL	5% BSA in TBS-T	1 in 1000			0.1 % BSA in 1X PBS	1 in 100
PepT1	Goat		sc-19917 / Santa Cruz / 200 µg / mL			0.2 % BSA and 0.5 % Triton-X in 1X PBS	1 in 100		
ERK	Rabbit	44 kDa	sc-93 / Santa Cruz / 200 µg / mL	5% Milk in TBS-T	1 in 1000				
ATPase Na^+ / K^+ beta 3	Mouse	31 kDa	H00000483-B01P Novis Biologicals / 0.5 mg / mL	5% Milk in TBS-T	1 in 500				
Rabbit mAB isotype control	Rabbit		3900 / Cell Signalling / 2.5 mg / mL					0.1 % BSA in 1X PBS	1 in 100
Mouse (G3A1) mAB isotype control	Mouse		5415 / Cell Signalling / 2.5 mg / mL					0.1 % BSA in 1X PBS	1 in 100
Beta Actin	Rabbit	45 kDa	4967 / Cell Signalling / 0.2 mg / mL	5% Milk in TBS-T	1 in 500				
YAP	Rabbit	65 kDa	sc-15407 / Santa Cruz / 200 µg / mL	5% Milk in TBS-T	1 in 1000				
Secondary antibodies									
FITC-conjugated anti Rabbit	Goat		FI-1000 / Vector / 1.5 mg / mL			0.2 % BSA and 0.5 % Triton-X in 1X PBS	1 in 400		
FITC-conjugated anti Goat	Rabbit		FI-5000 / Vector / 1.5 mg / mL			0.2 % BSA and 0.5 % Triton-X in 1X PBS	1 in 400		
Texas Red-conjugated anti Mouse	Horse		TI-2000 / Vector / 1.5 mg / mL			0.2 % BSA and 0.5 % Triton-X in 1X PBS	1 in 400		
HRP - conjugated anti Rabbit	Donkey		NA934 / GE Healthcare / 200 µg / 0.5 mL	TBS-T	1 in 1000				
HRP- conjugated anti Mouse	Donkey		NXA931 / GE Healthcare / 200 µg / 0.5 mL	TBS-T	1 in 1000				

Chapter 3

3 PepT1 as a Drug Delivery Target to Treat Chronic Pancreatitis and Pancreatic cancer

PepT1 as a Drug Delivery Target to Treat Chronic Pancreatitis and Pancreatic cancer

3.1 Background

Most cells in the body express transporter systems capable of selectively shuttling molecules that otherwise would be impermeable to the plasma membrane. Since the majority of these materials brought into cells via specialised transporter systems are nutrients, many of these integral membrane proteins are constitutively expressed at the cell surface of these cells. Nonetheless, the expression profile of a few transporters can be restricted to specific conditions or are cell type dependent (Cravo and Mrsny, 2013). Carrier-mediated transport, either Na^+- or H^+- coupled, can play an active role in the transport of drugs (Tsuji and Tamai, 1996). PepT1 in particular, represents an attractive strategy for pro-drug design as it opens up the possibility of chemically coupling a drug with a nutrient moiety, which could increase the efficacy of its uptake in cells that express that transporter (Lee, 2000).

As previously mentioned, Gonzalez and co-workers reported high expression of the oligopeptide transporter PepT1 in two human PDAC cell lines, AsPc-1 and Capan2 with a ductal origin and a *K- Ras* genotype (Gonzalez et al., 1998). This finding has fuelled interest in adopting PepT1 as a possible drug delivery strategy to target PDAC. Since PDAC cells may overexpress this transporter system in tumours and non-cancerous pancreatic cells do not, this occurrence may provide a novelty in tumour targeting strategy. Enhanced peptide transporter activity has also been reported in gastric cancer, osteosarcoma, bladder cancer and cholangiosarcoma (Inoue et al., 2005; Knutter et al., 2002). In addition, Mitsuoka and co-workers used a radiolabelled peptide PET tracer ^{11}C-Gly-Sar, an well-established PepT1 substrate, as a tool for cancer detection *in vivo* (Mitsuoka et al., 2008). The approach of using Gly-Sar to reveal cancer cells that over-express PepT1 provides an exciting opportunity to identify tumours (Mitsuoka et al., 2008). Gly-Sar, as a well characterised

dipeptide substrate for PepT1 (Brandsch, 2009; Hu et al., 2008; Ocheltree et al., 2005; Thwaites et al., 1993), is peptidase and hydrolysis resistant as it contains a N-methyl amide isostere (Mitsuoka et al., 2010).

Through the adoption of rational drug design, a therapeutic compound could be masked with a nutrient moiety; in the case of PepT1 this is an amino acid that is used to generate a pro-drug. Being a low affinity and high substrate capacity system (Daniel, 1996; Steiner et al., 1995), specific molecules with very unique characteristics can be coupled to drugs in order to increase the specificity of the complex for PepT1 which would increase the efficiency of drug permeation through the biological barrier. Other advantages in designing drugs according to a specific drug transport pathway include not only the specific uptake, but also specialised drug delivery systems that target to specific drug transporters.

Previous studies have demonstrated that single amino acid pro-drugs of non-PepT1 substrates could potentially use this transporter system to enter the cell (Cao et al., 2012). Tsume and co-workers reported that amino acid ester pro-drugs of the anti-cancer agent floxuridine had enhanced cellular uptake and cytotoxic activity in PDAC cells; this approach also enabled a striking prolonged systemic circulation and consequently a more efficient therapeutic action (Tsume et al., 2008). Floxuridine however, is most often used in colorectal cancer treatment (Hohn et al., 1989). In what might be more directly relevant to PDAC, amino acid ester conjugates of gemcitabine have been evaluated for uptake by peptide transporters using HeLa cells stably expressing PepT1 as an *in vitro* model; this approach resulted in an improved drug delivery to cancer cells or tissues overexpressing PepT1 (Song et al., 2005).

PepT1 as a Drug Delivery Target to Treat Chronic Pancreatitis and Pancreatic cancer

The aims of this chapter were initially to validate the expression of PepT1 in pancreatic cell lines from normal and cancerous origin and to evaluate, for the first time, the distribution of PepT1 in human pancreatic biopsies from normal, inflamed (CP) and cancerous tissues (PDAC). Secondly, this work also aimed to explore the role of PepT1 in pancreatic cancer cell proliferation. Finally, this study assessed the feasibility in using this transporter system as a drug delivery strategy by comparing the uptake of chemically modified anti-cancer and anti-inflammatory agents in a novel system: AsPc-1 PepT1 expressing and AsPc-1 PepT1$^{-/-}$ cell line.

3.2 Materials and methods

3.2.1 Cell culture and immunostaining

AsPc-1 (passage 12), Capan2 (passage 12), HPAFII (passage 8) and HPDE - human pancreatic ductal epithelial cell line - (passage 3) were grown on coverslips reaching up to 50 % confluency (generally 2 days for AsPc-1 and Capan2 and 5 days for HPAFII and HPDE cells). After cells were fixed and permeabilised as described in section 2.9, Chapter 2, they were subjected to a 1 h incubation with a primary antibody, PepT1 (custom made antibody - Innovagen) at 4 °C. A polyclonal antisera raised against PepT1 was affinity purified and prepared by Innovagen from rabbits injected with a peptide corresponding to the last 18 amino acid residues of the human protein PepT1 (**LEKSNPYFMSGANSQKQM**) (Gonzalez et al., 1998). Afterwards, cells were washed and incubated with the secondary antibody (Table 2.6, Chapter 2). Subsequently, slides were mounted as described in section 2.9, Chapter 2 and then taken to confocal microscopy. PSC lysates were a kind gift from Dr. Mert Erkan from the Technical University of Munich; stellate cells were cultured and isolated by individuals in Dr. Erkan´s laboratory as previously described (Erkan et al., 2010).

3.2.2 Immunoprecipitation and Western Blot

For immunoprecipitation assay, Capan2, HPAFII and HPDE were used at passages 12, 8 and 4, respectively. Cells were lysed as mentioned in section 2.4. Whole cell lysates containing approximately 500 µg of protein were incubated with a pull-down antibody, PepT1 (Innovagen - Table 2.6, Chapter 2) overnight at 4 °C. Proteins were then isolated as described in section 2.6.2.2, Chapter 2. Isolates were analysed through Western Blot as outlined in 2.6.2.1, Chapter 2. For Cox-2 experiments, cells were exposed to the compounds 100 µM Ala(S)Asp-IB and 100 µM control ibuprofen (IB) for 24 h and then lysed for protein expression as described in section 2.4, Chapter 2.

3.2.3 Immunohistochemistry

Fifteen human biopsies from five different patients (five normal, five CP and five PDAC) were obtained as described in Erkan, *et al*. (Erkan et al., 2010). PepT1 tissue distribution levels were assessed. Paraffin tissue sections were prepared according to section 2.10 Chapter 2, using VECTASTAIN Elite ABC Kit (Rabbit IgG). For antigen detection, slides were subject to an overnight incubation in a humid chamber at 4 °C with the polyclonal antisera recognizing PepT1 or control antisera from a control Rabbit (Table 2.6, Chapter 2). After, slides were mounted on DePex, they were allowed approximately 12 h to dry; subsequently slides were examined as described in section 2.9, Chapter 2.

3.2.4 Generation of stable PepT1 knock-down cell line: AsPc-1 PepT1$^{-/-}$

AsPc-1 cells, passage 8, were infected with lentiviral particles (shPepT1-**GCGGCTCATCTCCCAAATT**) as outlined in section 2.11, Chapter 2. After selecting the

colonies of interest, cells were passaged and trypsinized according to section 2.1, Chapter 2. To confirm the down-regulation of PepT1 signal at the membrane (PepT1$^{-/-}$), cell fractionation of AsPc-1 lysates was performed according to section 2.4, Chapter 2. Lysates were then subject to Western Blot (Section 2.6, Chapter 2). For immunostaining analysis, AsPc-1 WT, wild type, (passage 14) and AsPc-1 PepT1$^{-/-}$ (passage 2) were grown on coverslips for approximately two days to reach approximately 70 % confluence. Cells were then fixed and permeabilised as described in section 2.9, Chapter 2. For co-localisation purposes, cells were simultaneously incubated with the membrane marker E-cadherin and the antibody PepT1 (Table 2.6, Chapter 2) at 1:100 dilution for 1 h at RT. Subsequently cells were exposed to rabbit secondary antibody for 1 h and then to DAPI for 20 minutes, as described in section 2.9, Chapter 2. After being mounted in mowiol, slides were examined through confocal microscopy and the image analysed through the software Start LSM 5 Image Browser.

3.2.5 Cell treatment with different compounds and cell viability/proliferation assays

AsPc-1 WT and AsPc-1 PepT1 $^{-/-}$ were plated in 96 well plates at a cell density of 500 cells per well. Six different testing concentrations of BCH (2-Amino-2-norbornanecarboxylic acid), reported to be an L-system amino acid transporter inhibitor (Kim et al., 2008; Mitsuoka et al., 2010), were used: 0.3 mM, 1 mM, 3 mM, 10 mM, 20 mM and 50 mM. Test compound was applied to the cells as described in section 2.8, Chapter 2 and left incubating for 24 h and 48 h. Subsequently, 20 µL of MTS reagent was added per well and the plate returned to the incubator. After 3 h, absorbance readings were taken at 490 nm. Data was plotted using the software Graphpad Prism 5 software.

Gemcitabine and ibuprofen chimeras were obtained from Prof. Patrick Bailey and Gayle Wilson, University of Keele, UK. Compounds were dissolved in DMSO to a final

concentration of 50 mM. Eight different testing concentrations were further prepared: 500 µM, 200 µM, 100 µM, 50 µM, 20 µM, 10 µM, 5 µM and 1 µM. After incubating the cells with the compounds for the chosen exposure time, cell viability and cell proliferation was assessed using either the MTS or CyQuant reagent, as described in section 2.8 and 2.7 respectively, Chapter 2. To evaluate the involvement of PepT1 in mediating the uptake of chemically modified gemcitabine and ibuprofen, cell culture media was either supplemented with PepT1 substrate, 50 mM Gly-Sar (Brandsch, 2009; Hu et al., 2008; Ocheltree et al., 2005; Thwaites et al., 1993) or with the L-system amino acid transporter inhibitor, 10 mM BCH (Mitsuoka et al., 2010).

3.2.6 HPAF II and Caco 2 treatment with TNF α and IFN γ;

HPAFII and Caco 2 were grown on trans-wells for 14 days. TERs were checked on a daily basis and on day 14, HPAFII and Caco 2 acquired a TER value of typically 600 $\Omega.cm^2$ and 300 $\Omega.cm^2$, respectively. On day 10, the FBS was increased to 30 % (vol/vol) to induce apical Na^+/H^+ exchange activity as described in Vavricka´s study (Vavricka et al., 2006). 50 ng/mL of interferon γ (IFN γ) or tumour necrosis factor α (TNFα), were added to the basolateral compartment and left incubating for 2, 24 and 48 h. The apical compartment was acidified with a specific buffer in order to increase the activity of PepT1 due to additional protons, which are co-transported with PepT1 substrates. The acidified apical flux buffer contained 15 mM MES, pH 6.5, 140 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 5 mM KCl. The basolateral side buffer consisted of 130 mM choline chloride, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 15 mM HEPES, pH 7.4 and 20 mM NaCl (Vavricka et al., 2006). PepT1 protein levels were determined through Western Blot as described in section 2.6, Chapter 2.

3.3 Results

3.3.1 Validation of PepT1 expression and localisation in human PDAC cell lines compared to a normal ductal cell line

For the validation of PepT1 expression in PDAC, three different cell lines were used. Capan2 cell line is derived from a 56-year old male Caucasian and is from pancreatic ductal origin. These cells grow in adherent tissue culture and display epithelial features (Kyriazis et al., 1986). AsPc-1 cells were initially isolated from ascites fluid and demonstrate aggressive growth characteristics and metastatic behavior; these cells fail to differentiate (apical-basal polarization) when grown *in vitro* (Tan and Chu, 1985). HPAFII cell line, established from a human pancreatic adenocarcinoma, can differentiate *in vitro* to form polarised monolayers on permeable supports (Kim et al., 1989). For localisation studies, we have also used HPDE cells isolated from primary and immortalized epithelial cell lines of human pancreatic ducts (Furukawa et al., 1996). Firstly, PepT1 was precipitated out of whole cell lysates from Capan2, HPAFII and AsPc-1 (Figure 3.1-A). PepT1 signal was detected in the three cell lines tested consistent with previous findings (Gonzalez et al., 1998). Subsequently, PepT1 localisation was assessed in HPDE, AsPc-1 and Capan2 cell lines through confocal microscopy (Figure 3.1-B). In HPDE cells, PepT1 was predominantly detected in the cytoplasm and a similar pattern was observed for HPAFII cells (data not shown). In AsPc-1, a clear ring shape could be observed suggesting that PepT1 was distributed mainly at the cell surface of these cells. In Capan2 cells, PepT1 localised at the cytoplasm and more predominantly at the cell membrane. HPAFII cell line expressed the highest level of PepT1 whereas HPDE cell line showed the lowest level (Figure 3.1-C). Since AsPc-1 cells expressed PepT1 at a significant higher level compared to HPDE and the oligopeptide appeared to be distributed exclusively at the cell membrane,

in contrast to Capan2, AsPc-1 cells were selected for the *in vitro* model to carry out functional studies.

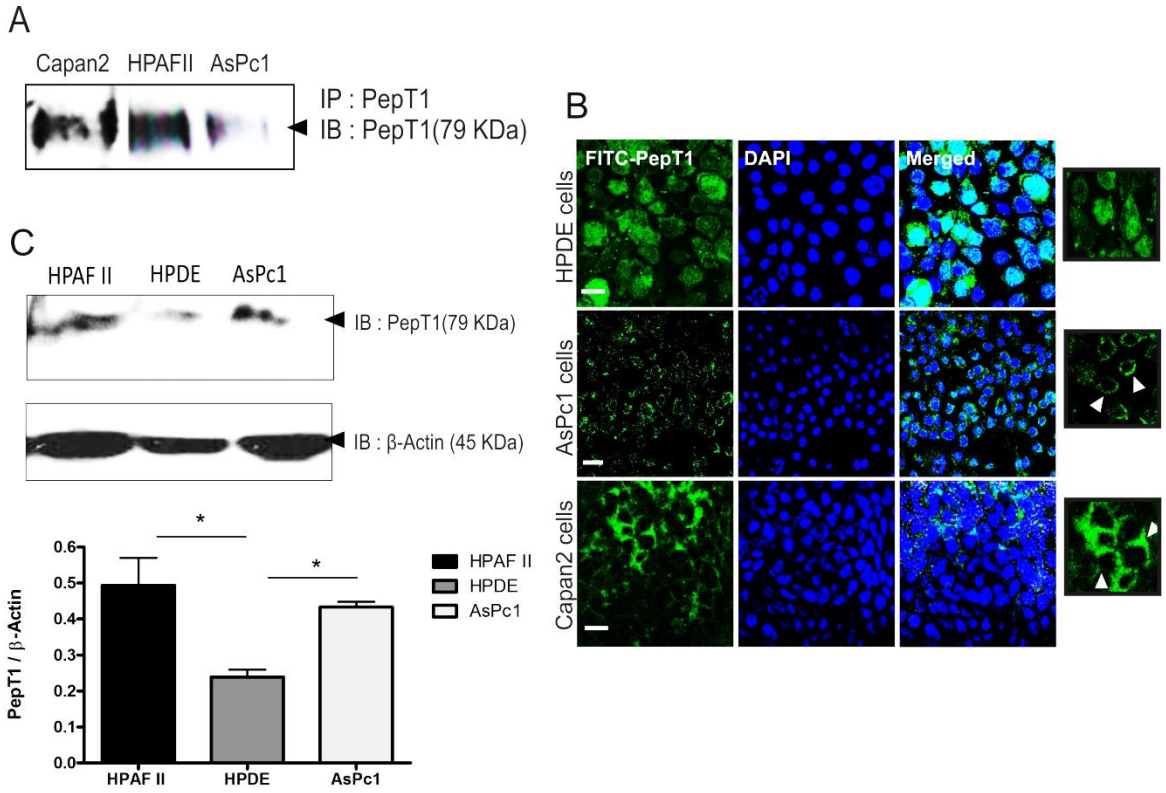


Figure 3.1 PepT1 is expressed in PDAC derived cell lines and localizes at the cell surface; **(A)** Immunoprecipitation of PepT1 in whole cell lysates from three cell lines derived from PDAC patients; **(B)** Confocal microscopy images showing PepT1 distribution in HPDE cells (top panel) in AsPc-1 cells (middle panel) and in Capan2 cells (bottom panel); Scale bar, 20 µm. Magnified micrographs of FITC-PepT1 on the right hand side of the panel; **(C)** Immunoblot comparing PepT1 expression levels in whole cell lysates from three different pancreatic cell lines. Data is presented as the mean of 3 independent experiments ± SEM; *p < 0.05 compared to HPDE cell line.

PDAC is often accompanied by a vigorous stromal response, termed desmoplasia (Feig et al., 2012). From a drug delivery perspective, this fibrotic component assumes great importance as the microenvironment established around the organ works as a physical barrier to chemotherapeutic events, preventing efficient pancreatic drug perfusion (Jaffee et al., 2002). As previously mentioned, PSCs play a major role in the pathobiology of CP and PDAC contributing to the remarkable fibrosis observed in the exocrine pancreas (Omary et al., 2007). We sought to analyse PepT1 expression levels in PSCs lysates from

three patients with PDAC and two with CP. PepT1 signal was detected in the five samples analysed, regardless the pathological condition (Figure 3.2).

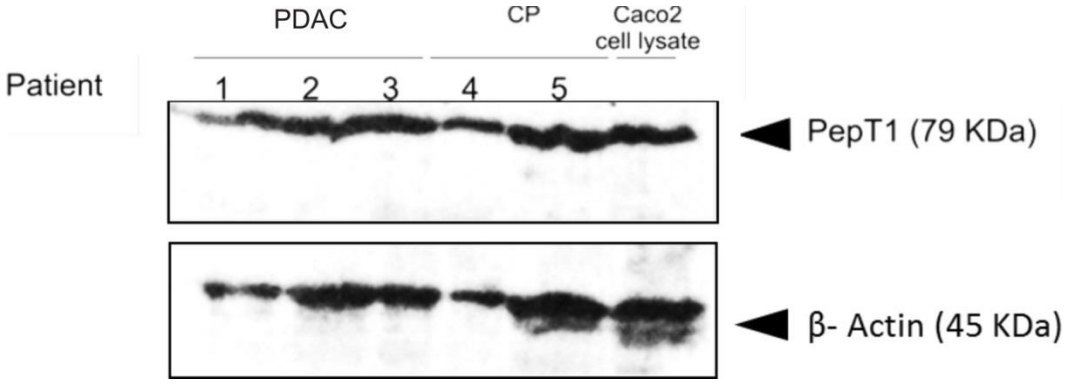


Figure 3.2 PepT1 is expressed in PSC from patients diagnosed with PDAC and CP. Immunoblot showing PepT1 expression in pancreatic stellate cell lysates from three patients diagnosed with PDAC and two with CP and in whole cell lysates from Caco 2 cells used as a control. Data presented as n=1 due to limited sample volume.

3.3.2 Assessment of PepT1 distribution in normal, inflamed and cancerous human pancreatic tissue

While PepT1 expression and localisation has been described in pancreatic cancer cells (Gonzalez et al., 1998), no studies have previously reported PepT1 distribution in pancreatic tissue. Through a collaboration established with Dr. Mert Erkan, pancreatic biopsies were obtained from healthy individuals and patients that had been diagnosed either with CP or PDAC.

Each slide contained two paraffin embedded tissue sections. One section was exposed to the antisera raised against a human PepT1 peptide while the other section was exposed to control antisera (Table 2.6, Chapter 2) to estimate the extent of non-specific antibody binding due to Fc (fragment crystallisable of the antibody) receptor binding or other protein-protein interactions that could compromise labelling specificity. Because no staining was obtained for the tissue sections treated with the control antisera, as shown by the

insets, PepT1 staining was positively validated (Figure 3.3); (Figure 8.4, Chapter 8). PepT1 distributed at the cell surface of inflamed and cancerous pancreatic ducts (Figure 3.3) and the staining intensity obtained was comparable across the different stages of cancer cell differentiation or TNM (tumour, nodes and metastasis) staging (Table 3.1).

PepT1 as a Drug Delivery Target to Treat Chronic Pancreatitis and Pancreatic cancer

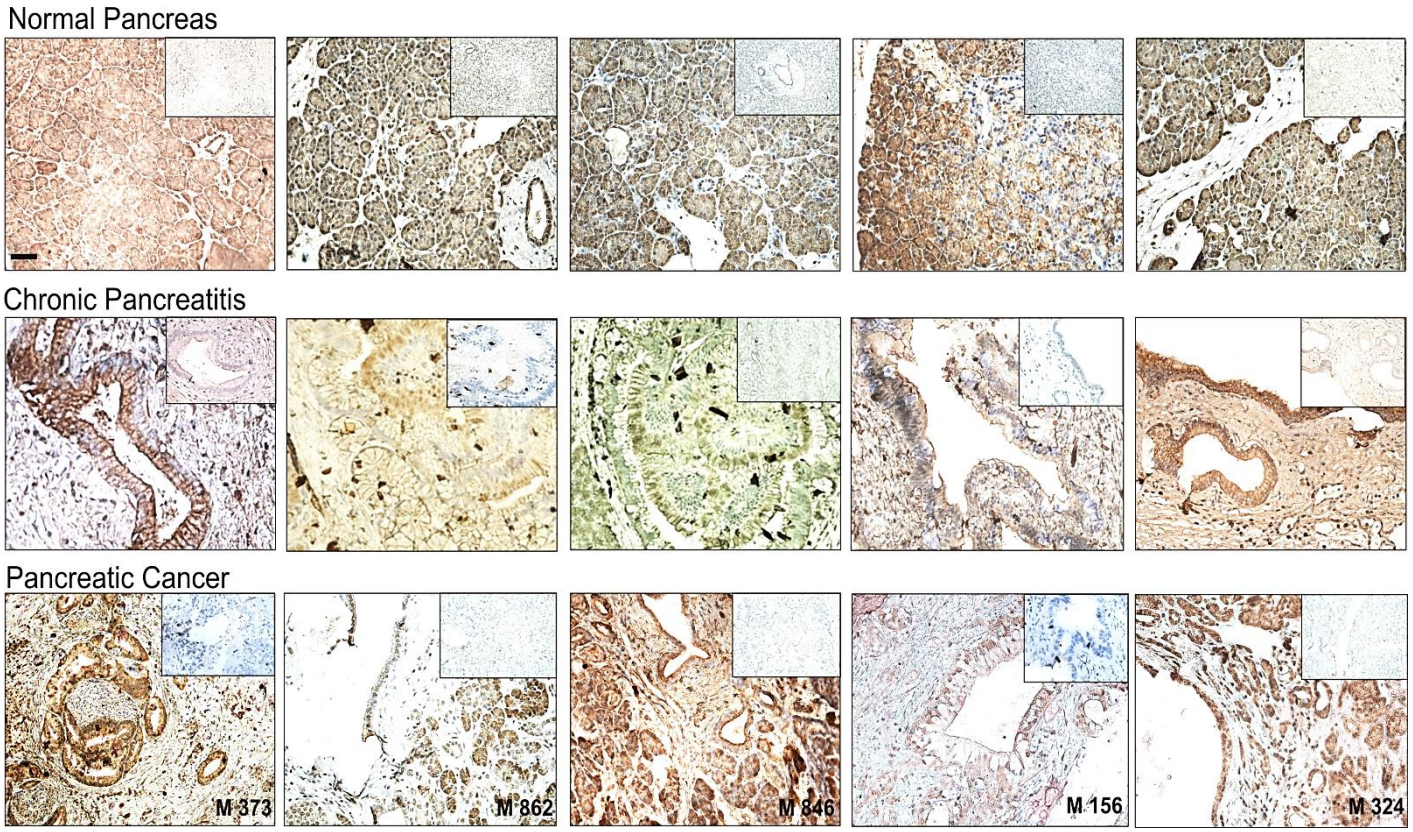


Figure 3.3 PepT1 distributes at the cell surface of morphologically inflamed and cancerous pancreatic ducts. PepT1 expression in five different patients diagnosed with CP and PDAC compared to normal pancreas. Scale bar, 50 µm.

Table 3.1 Table showing the medical information concerning the nature and aggressiveness of PDAC in five different patients.

Patient ID	Age	Gender	TNM	Grade	Resection stage	Survival Time (Months)	Status
M373	68	F	$T_3N_1M_1$	G_3	NA	NA	NA
M862	67	F	$T_3N_0M_0$	G_3	R0	NA	NA
M846	77	F	$T_3N_0M_0$	G_3	NA	36.7	Alive
M156	75	F	$T_3N_0M_0$	G_1	R0	60	Life
M324	63	M	$T_3N_1M_0$	G_2	R0	12.5	Alive

Abbreviations: **T_3** - The cancer has spread from the pancreas to surrounding tissues near the pancreas but not to major blood vessels or nerves; **T_4** - The cancer has grown further outside the pancreas, into the nearby large blood vessels; **N_0** – tumor cells absent from regional lymph nodes; **N_1** – Regional lymph node metastasis are present; **M_0** - No distant metastasis; **M_1** - metastasis to distant organs (beyond regional lymph nodes); **G_1** - Well differentiated (Low grade) ; **G_2** - Moderately differentiated cancer cells; **G_3** - Poorly differentiated (High grade) ; **R_0** - No residual tumour; **R_1** – Microscopic residual tumor.

PDAC develops through a multistep progression model; the ducts are the first structures to become morphologically altered which are subject to non-invasive intra-ductal lesions culminating in invasive adenocarcinoma (Basturk et al., 2010). Being the structures more prone to malignancy, PepT1 distribution was closely examined in altered ductal structures and compared to normal pancreatic ducts from different patients.

PepT1 localised essentially at the cytoplasm of normal acinar cells and negligible staining was observed in ductal structures from normal pancreas. In cancer, however, PepT1 was distributed primarily at the apical surface of poorly- and well-differentiated pancreatic ducts (as indicated by the black arrows in Figure 3.4). This finding provides support to the strategy of directing the uptake of chemically modified compounds into PepT1 substrates to tumour cells. Moreover, the differential distribution between normal and cancerous ductal structures should not only allow for an efficient drug delivery but would also confer a certain degree of

selectivity. Yet, it is rather premature to make definite assumptions given the small sample size analysed as only six patients were studied in this work.

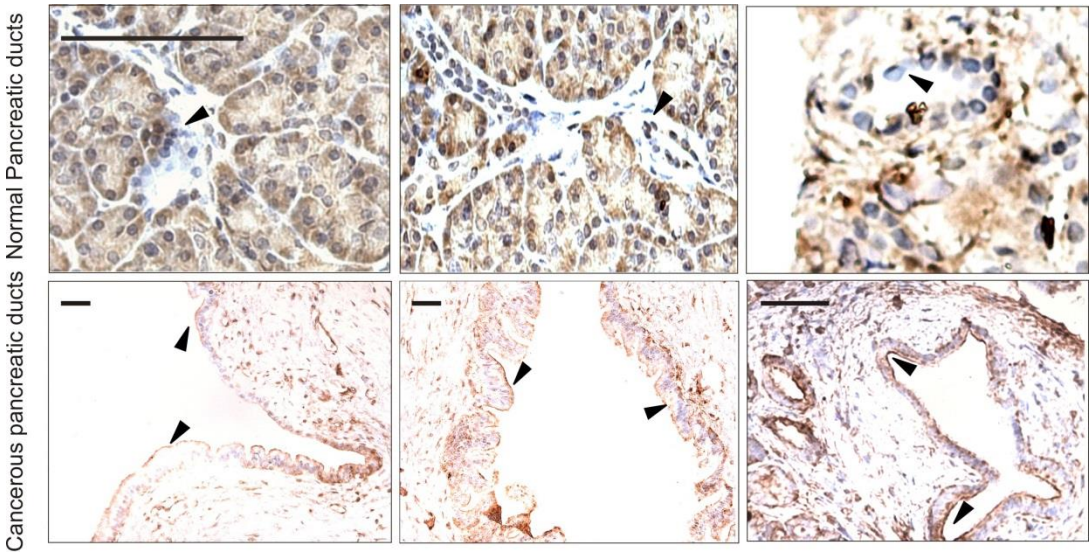


Figure 3.4 PepT1 signal at the cell surface of malignant transformed pancreatic ducts is stronger compared to morphologically normal ducts. PepT1 distribution levels in normal (top panel) and cancerous pancreatic ducts (bottom panel) from six different patients.Scale bar, 50 µm

3.3.3 Generation of a knockout PepT1 cell line through lentiviral particles

PepT1 deficient mice have already been generated and were found to be viable, fertile, grew to normal size and weight, and showed no obvious abnormalities (Hu et al., 2008). In order to assess the functionality of PepT1 in cancerous cells, a control cell line stably expressing reduced or absent levels of PepT1 had to be generated. This was achieved through targeting shRNA against a specific region in the PepT1 gene (**GCGGCTCATCTCCCAAATT**). This approach was repeated for two other PDAC derived cell lines expressing PepT1, namely Capan2 and HPAFII, but without success. To confirm PepT1 knock-down in the AsPc-1 cell line, localisation and expression assays were carried out (Figure 3.5). Confocal microscopy demonstrated some co-localisation between PepT1 and E-cadherin, an epithelial membrane marker of AJs, in AsPc-1 WT cells (Nelson et al., 1990). Conversely, no co-localization was obtained between the two proteins

in the AsPc-1 PepT1$^{-/-}$ cell line, validating the successful knock-down of PepT1. To further confirm the confocal microscopy data, PepT1 levels were analysed in membrane fractions from both cell lines. When compared to the wild type cell line, PepT1 was not detected at the membrane of cell lysates from PepT1$^{-/-}$ cells. Interestingly, a weak signal for nuclear expression and slightly more pronounced labelling in the cytosolic fraction was observed in the knock-down cell line. This occurrence is not particularly relevant, as this study focuses on the transport of PepT1 substrates across the plasma membrane, where the loss of expression of this transporter was primarily observed.

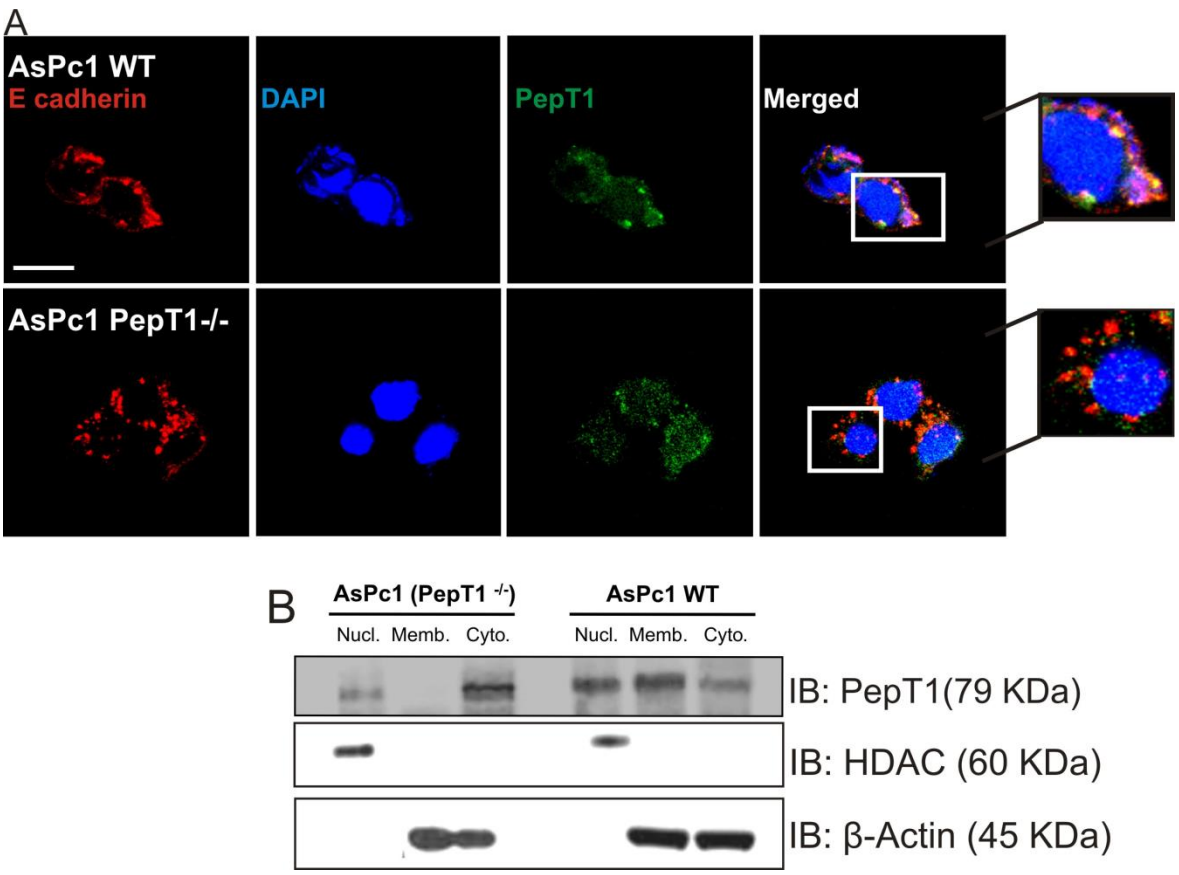


Figure 3.5 PepT1 knock-down in AsPc-1 cell line. **(A)** Confocal microscopy showing co-localisation of PepT1 and E-cadherin at the cell membrane of AsPc-1 WT; Scale bar, 20 µm; **(B)** Immunoblot analysis showing PepT1 expression levels at the cell membrane of AsPc-1 PepT1$^{-/-}$ cells compared to AsPc-1 WT.

3.3.4 Inhibition of PepT1 function decreased cell viability

Amino acids are organic compounds essential for protein synthesis which are critical for cell growth and proliferation in normal and transformed cells (Christensen, 1990). Amino acid transport across the plasma membrane is mediated by specialised amino acid transporter systems. Among these, are the system L amino acid transporters (LATs) responsible for Na^+ independent transport of neutral, branched or aromatic amino acids to the cells (Christensen, 1990).

BCH is a model compound for the study of amino acid transporters as it is a specific inhibitor for LAT; BCH has been shown to be cytotoxic due to its ability to deplete ATP (Babu et al., 2003; Kim et al., 2008). Several studies have reported the anti-cancer potential achieved by inhibition of LATs and an enhancement in the cytotoxic activity of several chemotherapeutic agents (Haining et al., 2012; Kim et al., 2008). Importantly, inhibition of LATs results in reduced nutrient supply leading to cell growth suppression (Kim et al., 2008). Inhibition of oligopeptide transporter activity has been shown to suppress the growth of AsPc-1 cells known to express PepT1 (Gonzalez et al., 1998; Mitsuoka et al., 2010). Hence, the functional role of the oligopeptide transporter in AsPc-1 cells was examined.

WT and $PepT1^{-/-}$ cell lines were exposed to different concentrations of BCH over 24 and 48 h. At 24 h, cell viability did not differ considerably between cells expressing WT and $PepT1^{-/-}$ cell lines. However, after 48 h, $PepT1^{-/-}$ cell line exhibited less of an effect of BCH on cell viability compared to WT cells (Figure 3.6). Reduction in cell proliferation resulting from BCH exposure has been previously reported (Kim et al., 2008). We now show that even though BCH inhibits Na^+ - independent transport of amino acids, it can also affect the cell viability of PepT1 expressing cells. These results are consistent with other studies, however it appears that BCH not only inhibits the transport of amino acids into the cancer

cells but it can also affect the transport of oligopeptides (Kim et al., 2008; Mitsuoka et al., 2010). Manipulation of system L activity has been suggested to have anti-cancer implications; the inhibition of LAT1 activity in tumour cells was proposed to be effective in suppressing tumour cell growth through essential amino acid deprivation in tumour cells (Kanai and Endou, 2001; Kim et al., 2002). Our data suggests that PepT1 might be a new target for suppression of pancreatic cancer cell growth.

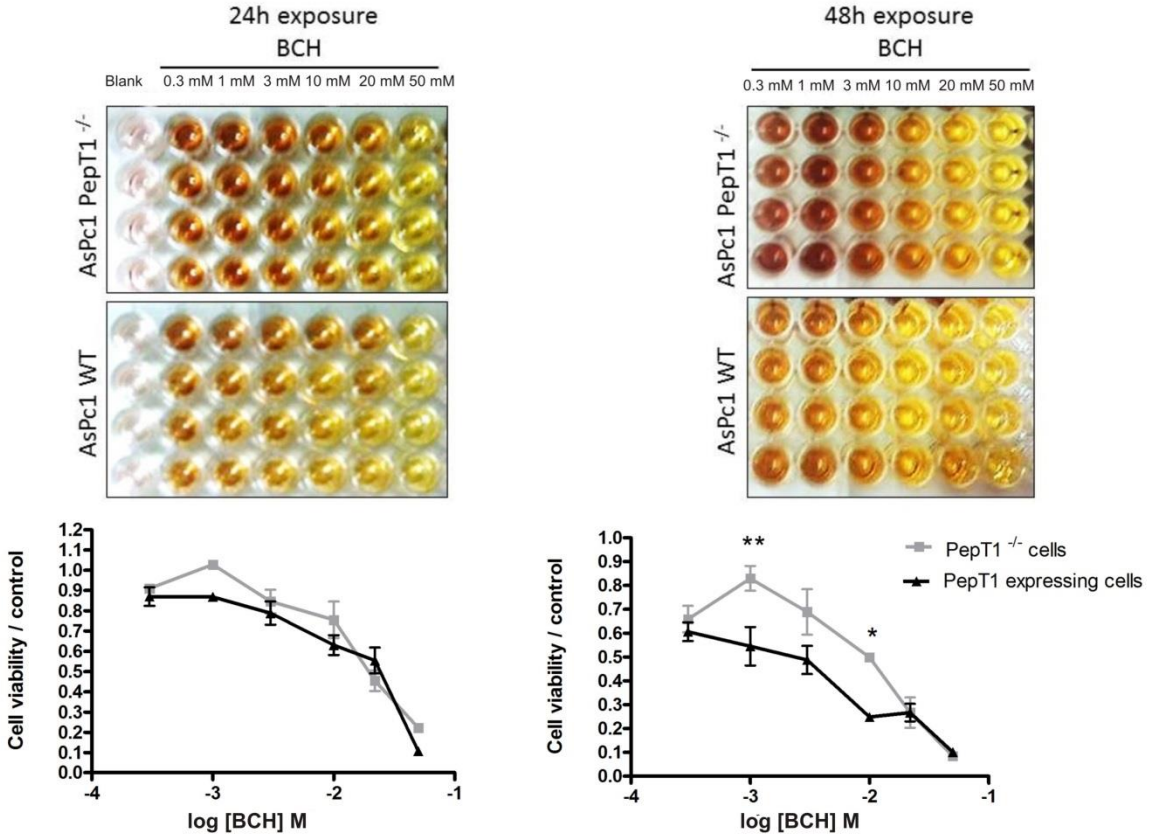


Figure 3.6 BCH decreases cell viability in PepT1 expressing cell lines in a more pronounced manner compared to the knock-down cell line. MTT assay in PepT1 expressing (AsPc-1 WT) and knock-down cell lines (AsPc-1 PepT1$^{-/-}$) after being exposed to different concentrations of BCH during a 24 h and 48 h time-course. Data is presented as the mean of 3 independent experiments ± SEM; *p<0.05;**p<0.01 compared to PepT1 expressing cell line.

3.3.5 Targeting PDAC through chemically modified PepT1 substrates

Gemcitabine, through its ability to incorporate into the genome and block DNA synthesis(Plunkett et al., 1995) , is a current front line treatment for pancreatic cancer (Burris et al., 1997). Despite being clinically effective, the drug has many side effects. Hence, conjugation of an amino acid moiety to gemcitabine structure could increase the selectivity of delivering this cytotoxic agent into the cytoplasm of cells overexpressing PepT1, such as those derived from PDAC patients. Despite the fact that the crystal structure of PepT1 is not yet available, PepT1 seems to be a more robust target to the cytotoxicity of chemotherapeutic agents than other membrane bound proteins (Lee, 2000).

Designing drugs in a way that favours their cellular uptake via membrane transporters provides an attractive approach to selectively targeting cancer cells. Such an approach has several advantages to the parent drug, including improved specificity of drug uptake and retention in the cytoplasm of cells that over-express a particular membrane transporter (Lee, 2000). Understanding the physical and chemical properties of the PepT1 transporter may improve the delivery of drugs via this route.

Despite the wealth of information regarding the prediction of a compound´s affinity for PepT1, little is known about its transport capacity. Meredith and co-workers, reported the ability of PepT1 in transporting thio-dipeptides pro-drugs of ibuprofen (Foley et al., 2009). Through a collaboration with the Bayley group at the University of Keele, the anti-cancer drug gemcitabine was chemically modified and linked to a hydrolysis resistant thio-dipeptide that is recognized as a potential substrate for PepT1. The carrier Ala(S)Asp- (Figure 3.7 - compound A) was attached to gemcitabine through the primary alcohol via an ester linkage. Gemcitabine is set slightly away from the carrier in the chimera that it could be placed, a feature identified to improve transport efficacy by Bayley´s group (data not

published). An important factor determining the sensitivity of a particular cell line to gemcitabine treatment is the intracellular retention of dFdCTP (Huang et al., 1991). Gemcitabine can be deaminated to form 2,2-difluorodeoxyuridine. The efficacy of formulated gemcitabine pro-drugs and consequently their transport across cells partially relies on the resistance to such enzymatic degradation at the cell surface (Song et al., 2005; Tsume et al., 2014). To overcome this, gemcitabine was attached to two carriers: 5-hydroxymethyl-ala(S)7-asp-gemcitabine (compound B) and 4-hydroxy-ala(S)asp-5-hydroxymethyl-ala(S)7--asp-gemcitabine (compound C).

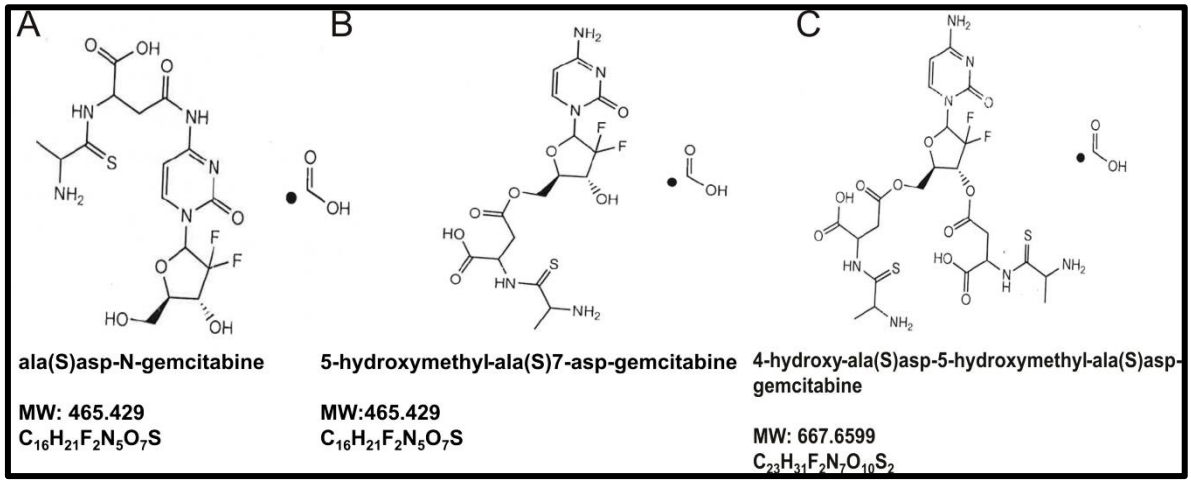


Figure 3.7 Gemcitabine modified chimeras as PepT1 substrates. Courtesy of Prof. Bailey and Gayle Wilson (University of Keele).

Firstly, we wished to characterise the cell viability response by directing the uptake of the three chimeras through the transporter system using as an *in vitro* model a PepT1 expressing and a PepT1$^{-/-}$ cell line. Unexpectedly, compound C was the most effective at decreasing AsPc-1 cell viability. Despite the fact that compound C had the highest molecular weight and according to the oocyte data showed the least efficacy in getting transported through PepT1 (data not shown), it was the most efficient chimera at reducing cell viability in PepT1 expressing compared to PepT1$^{-/-}$ cells (Figure 3.8).

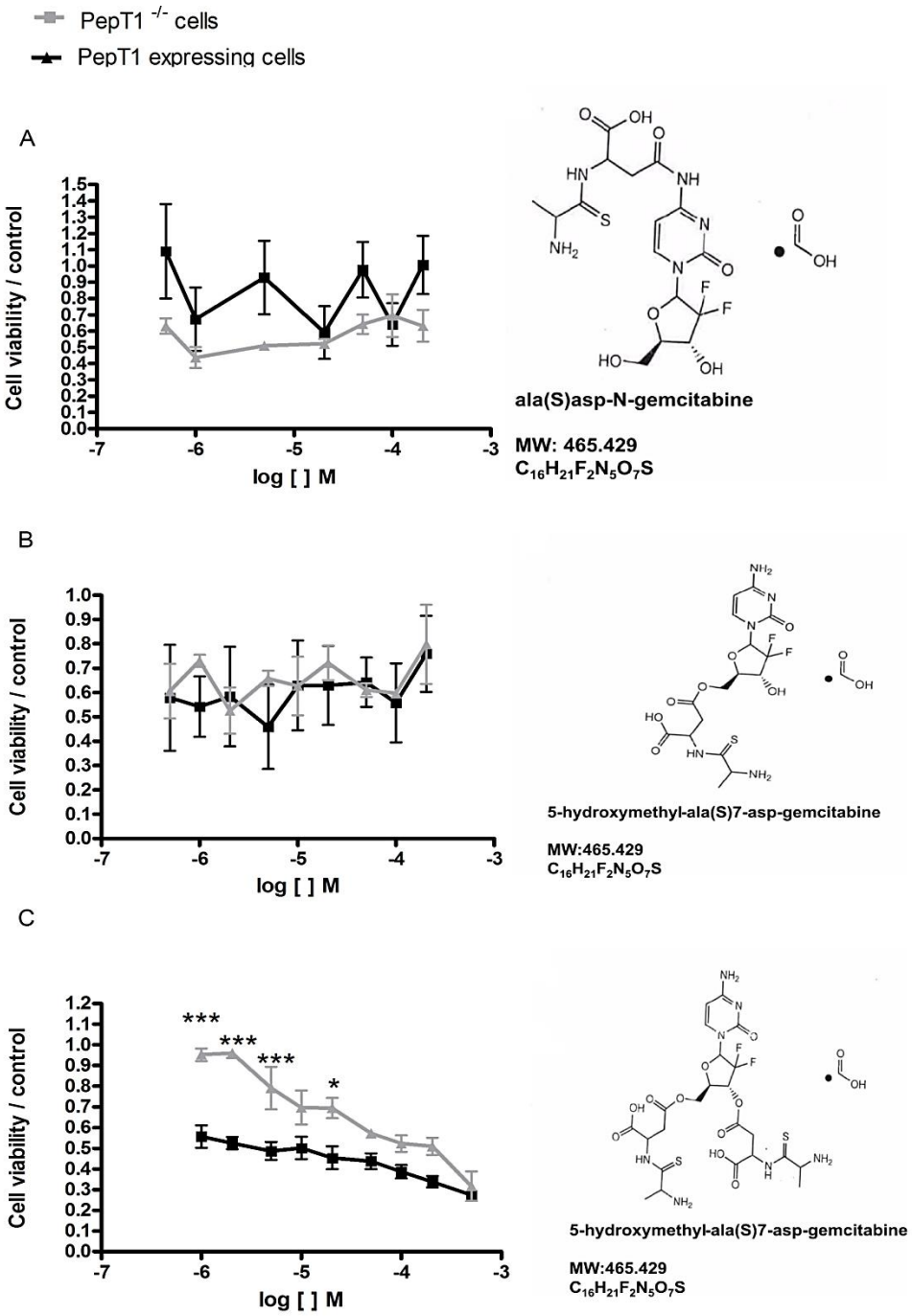


Figure 3.8 Compound C was the most effective gemcitabine modified chimera in decreasing cell viability mediated through PepT1 uptake. Concentration-response of modified gemcitabine compounds exposed to WT and PepT1 knock-down AsPc-1 cells. Data presented as mean of 3 independent experiments ± SEM; * $p < 0.05$; *** $p < 0.001$.

Cell viability was assessed after PepT1 expressing and PepT1$^{-/-}$ cell lines were exposed to compound C (Figure 3.9). The calculated EC_{50} in the cell line expressing PepT1 was nearly fivefold lower (~11 μM) than in the PepT1$^{-/-}$ cell line (~54 μM) suggesting the gemcitabine chimera was more efficient at reducing cell viability in the cell line expressing PepT1.

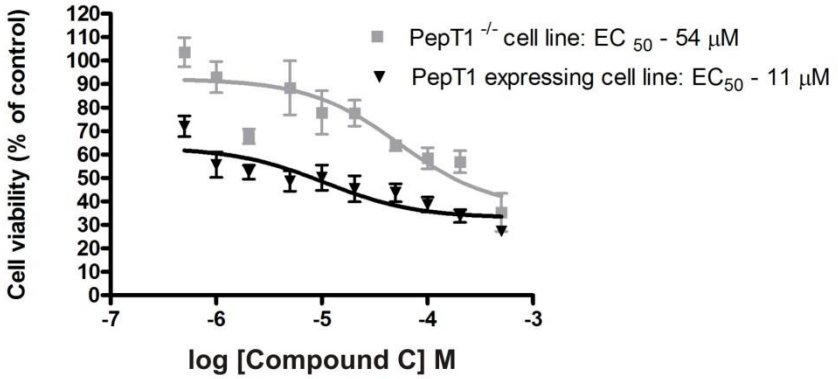


Figure 3.9 Compound C has a higher EC_{50} in the PepT1$^{-/-}$ cell line compared to the WT. Exposure of PepT1 expressing cell line and PepT1$^{-/-}$ to compound C over a 48 h period. Data presented as mean of 3 independent experiments.

Cell proliferation was assessed in PepT1 expressing and PepT1$^{-/-}$ cell line after exposure to compound C (Figure 3.10). There was a statistically significant decrease in cell proliferation in AsPc-1 cells when compared to cells with reduced levels of PepT1 expression (Figure 3.10). The selective growth inhibition of PepT1 expressing cells over PepT1$^{-/-}$ cells after exposure to the chemically modified gemcitabine compound suggests that it is mediated through PepT1.

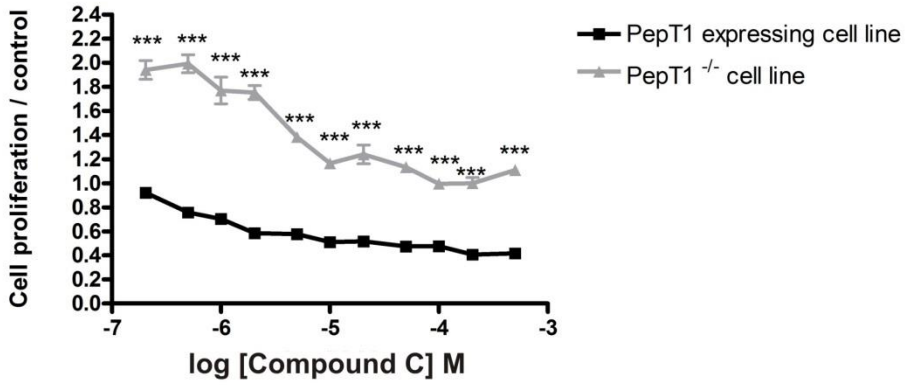


Figure 3.10 Compound C reduced cell proliferation in PepT1 expressing cells much more efficiently compared to the knock-down cell line. Cell proliferation rate of PepT1 expressing cells and knock-down cells after being exposed to compound C over a 48 h period. Data are presented as the mean of 3 independent experiments ± SEM; *** $p < 0.001$.

The cytotoxic potential of compound C was compared to gemcitabine. As it can be seen from Figure 3.11, cells treated with compound C had lower cell viability compared to the parent gemcitabine compound. Amino acids and mono-acid ester pro-drugs have been shown to be substrates for PepT1 (Landowski et al., 2005). The current results show that through a thio-dipeptide carrier the uptake of an anti-cancer agent through PepT1 can be improved.

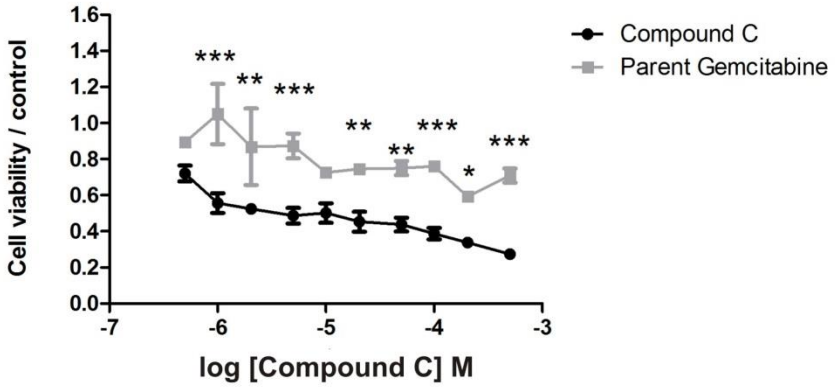


Figure 3.11 Compound C decreased cell viability more efficiently than its parent compound. AsPc-1 cells were exposed to compound C and to the parent compound over a 48 h period. Data presented as mean of 3 independent experiments ± SEM; *p < 0.05; ** p < 0.01; *** < 0.001.

Next, the specificity of PepT1-mediated transport involved in the uptake of compound C was investigated. To check this, PepT1 function was competed with using the specific substrate Gly-Sar (Brandsch, 2009; Hu et al., 2008; Ocheltree et al., 2005; Thwaites et al., 1993). Cells treated with normal media supplemented with compound C and 50 mM Gly-Sar showed a higher degree of cell viability compared to cells that were cultured in normal media with compound C only (Figure 3.12). Even though a statistically significant reduction in cell viability was only obtained for one specific concentration (1 μM), there was a trend suggesting that the improved cellular uptake of the gemcitabine chimera can be attributed to the oligopeptide transporter expressed in AsPc-1 cells.

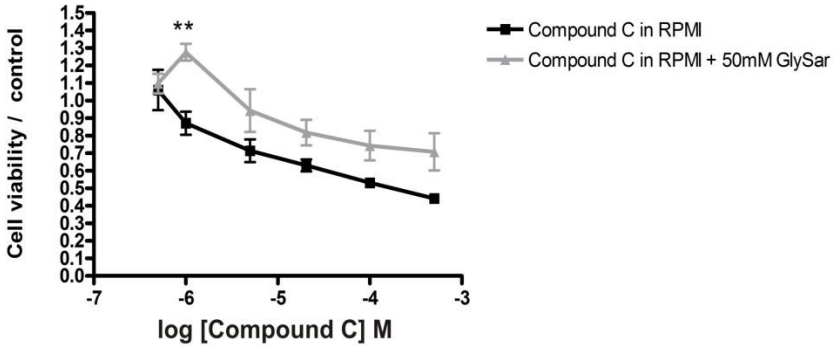


Figure 3.12 Cell viability showed a more pronounced decrease in cells treated with compound C compared to cells treated with compound C in a Gly-Sar enriched medium. AsPc-1 cells exposure to compound C in normal or Gly-Sar enriched medium over a 48 h period. Data presented as mean of 3 independent experiments ± SEM; ** $p < 0.01$.

After showing the potential effect of BCH in reducing AsPc-1 cell viability (Figure 3.6), it was investigated if by inhibiting amino acid transport there would be a difference in the uptake of compound C at different concentrations. Cell viability response did not differ greatly in response to compound C between the two cell lines when treated with 10 mM BCH, suggesting that PepT1, rather than LATs, is likely to be involved in compound C uptake (Figure 3.13).

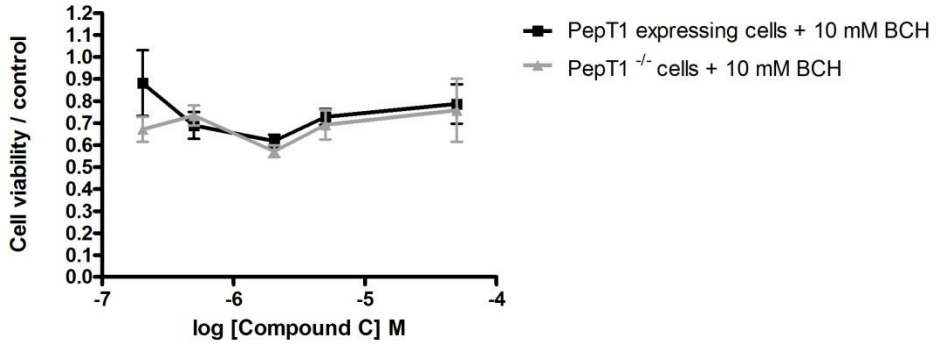


Figure 3.13 Similar cell viability response between PepT1 expressing and PepT1$^{-/-}$ AsPc-1 cells treated with compound C in cell media enriched with BCH. PepT1 expressing cell line and PepT1$^{-/-}$ were exposed to 10 mM BCH and different concentrations of compound C over a 48 h period. Data presented as mean of 3 independent experiments ± SEM.

3.3.6 Redistribution of PepT1 in CP biopsies

CP has been suggested to be a pre-empting factor contributing to the development of PDAC (Howes and Neoptolemos, 2002). Given this, we sought to investigate if there was a continuum in PepT1 expression levels resulting from the progression of normal to inflamed pancreas culminating in cancer. As previously shown (Figure 3.3), PepT1 was detected at the cell surface of inflamed ducts in five patients. As shown by Figure 3.14, a schematic representation of PepT1 expression throughout normal, inflamed and cancerous pancreas, suggests that there is a continuum in PepT1 expression consistent with the representative pancreatic biopsies collected from different patients. These data are in line with other studies reporting the involvement of PepT1 in inflammatory processes (Ingersoll et al., 2012; Nguyen et al., 2009). Merlin and co-workers also suggested that PepT1 was expressed in inflamed colonic tissues, while Buyse and colleagues reported PepT1 capacity in transporting formyl-Met-Leu-Phe (fMLP), a known neutrophil attractant peptide of bacterial origin (Buyse et al., 2002; Merlin et al., 2001).

Representative pictures of different areas of pancreatic tissue were taken from normal, inflamed and cancerous pancreatic tissue (Figure 3.14). The cellular distribution of PepT1 in biopsies obtained from patients diagnosed with CP was altered from that observed in non-inflamed samples (Figure 3.15). In normal pancreas, PepT1 labelling was not as strong as it was observed in CP or PDAC samples. At the early stages of CP (PanIN-1A, PanIN-1B), as pointed by the black arrows, PepT1 distributed at the apical side of inflamed pancreatic ducts; at later stages of inflammation (PanIN-3), the oligopeptide transporter was no longer located selectively at the apical surface of cells, as in a normal polarizing cell condition, but instead it relocated around the entire cell membrane. Finally in cancer, PepT1

labelling was markedly strong in malignant transformed ducts and a visible stromal distribution could also be observed.

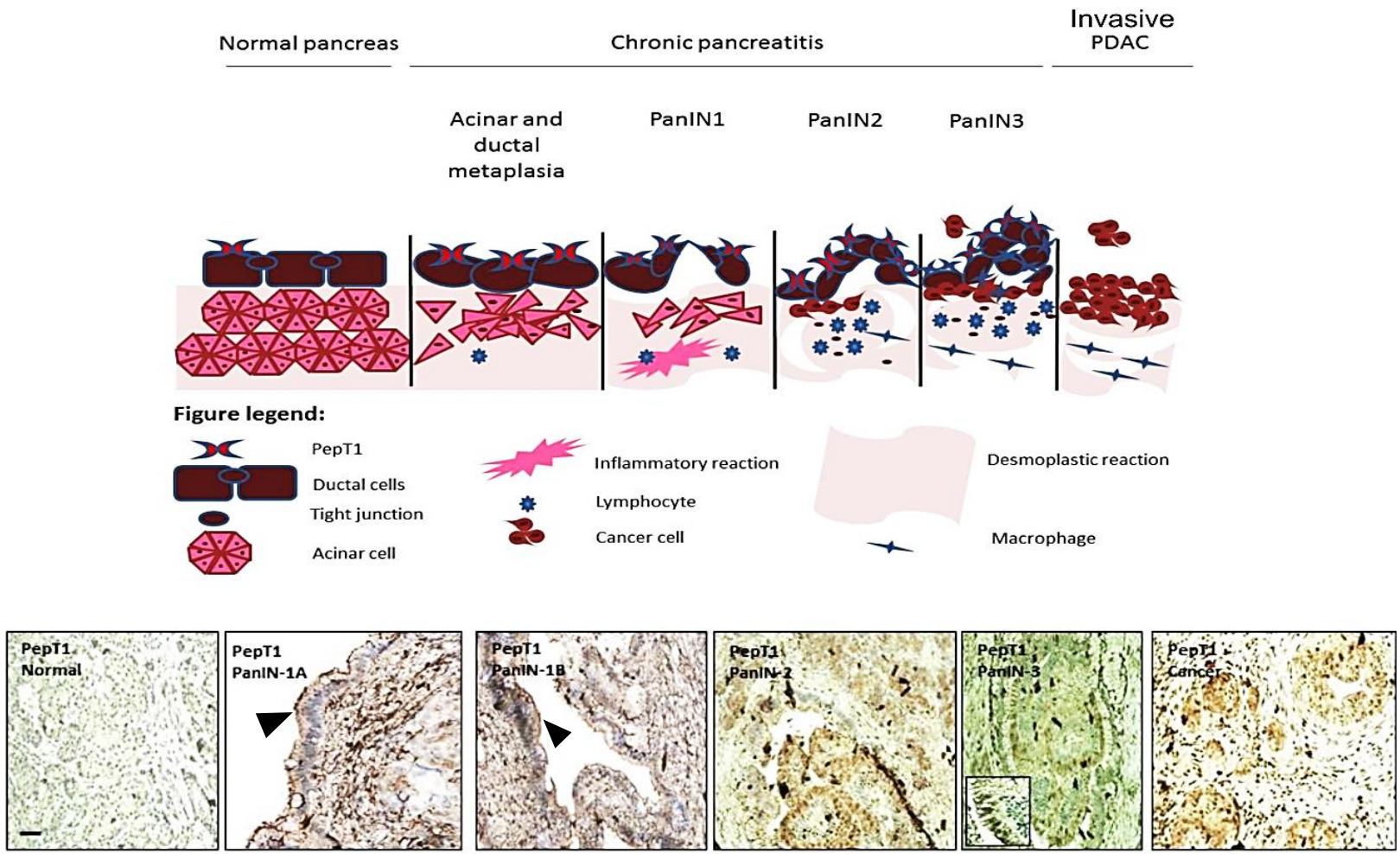


Figure 3.14 PepT1 expression in tissues from inflamed and cancerous pancreas compared to normal tissue. Diagram representing the changes in PepT1 expression levels in six different patient biopsies from normal, CP and PDAC tissue (scale bar, 50 µm).

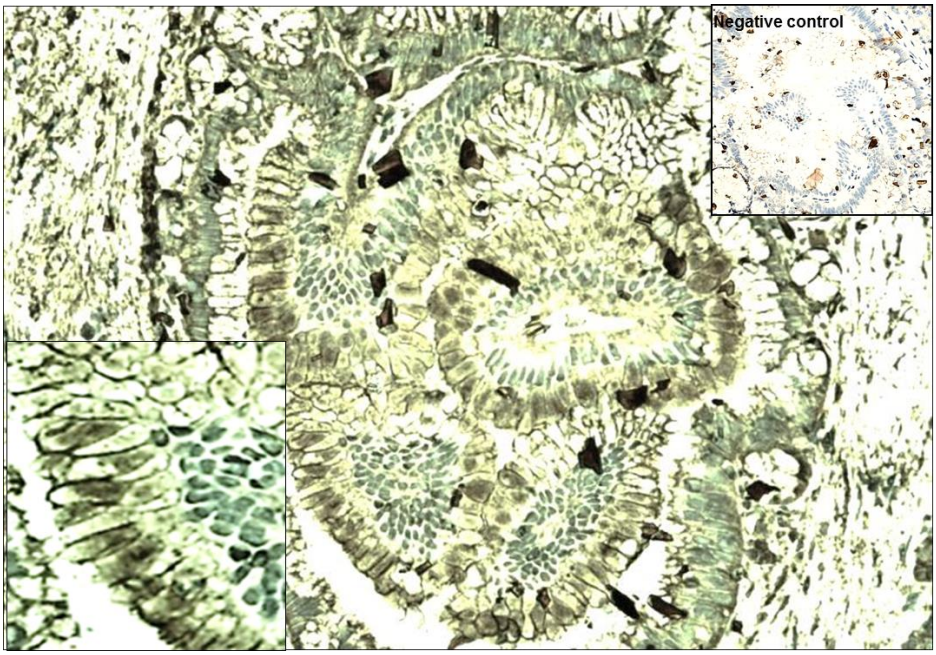


Figure 3.15 PepT1 redistributes at the cell surface of inflamed pancreatic cells. Biopsy sample collected from a patient diagnosed with CP and immunostained for PepT1.

3.3.7 The creation of an *in vitro* model of CP

Given the observed alteration of PepT1 distribution in CP, an *in vitro* model recapitulating the molecular biology of pancreatic inflammation would be very valuable, yet there are limited studies in this field. The up-regulation of PepT1 in intestine in response to cytokine exposure has been well documented (Buyse et al., 2003; Ingersoll et al., 2012; Vavricka et al., 2006; Wang et al., 2011). Moreover, a study reported that TNF α and IFN γ increased the activity, total and apical PepT1 expression in a concentration and time-dependent fashion in Caco 2 cells (Vavricka et al., 2006). However, the experimental setup referred in this study was not reproducible in the cell lines used for this study: HPAFII and Caco 2. Since both cell lines are able to polarise *in vitro* (Kim et al., 1989; Yee, 1997), an increase in PepT1 expression levels after cytokine induction would be expected; instead, PepT1 levels decreased after 24 h of cytokine treatment in HPAFII cells and in Caco 2 cells. Even though an increase was observed after 2 h of treatment, this cytokine treatment was unable

to sustain an increase in PepT1 expression levels after 24 and 48 h, so the experiment could not be validated (Figure 3.16).

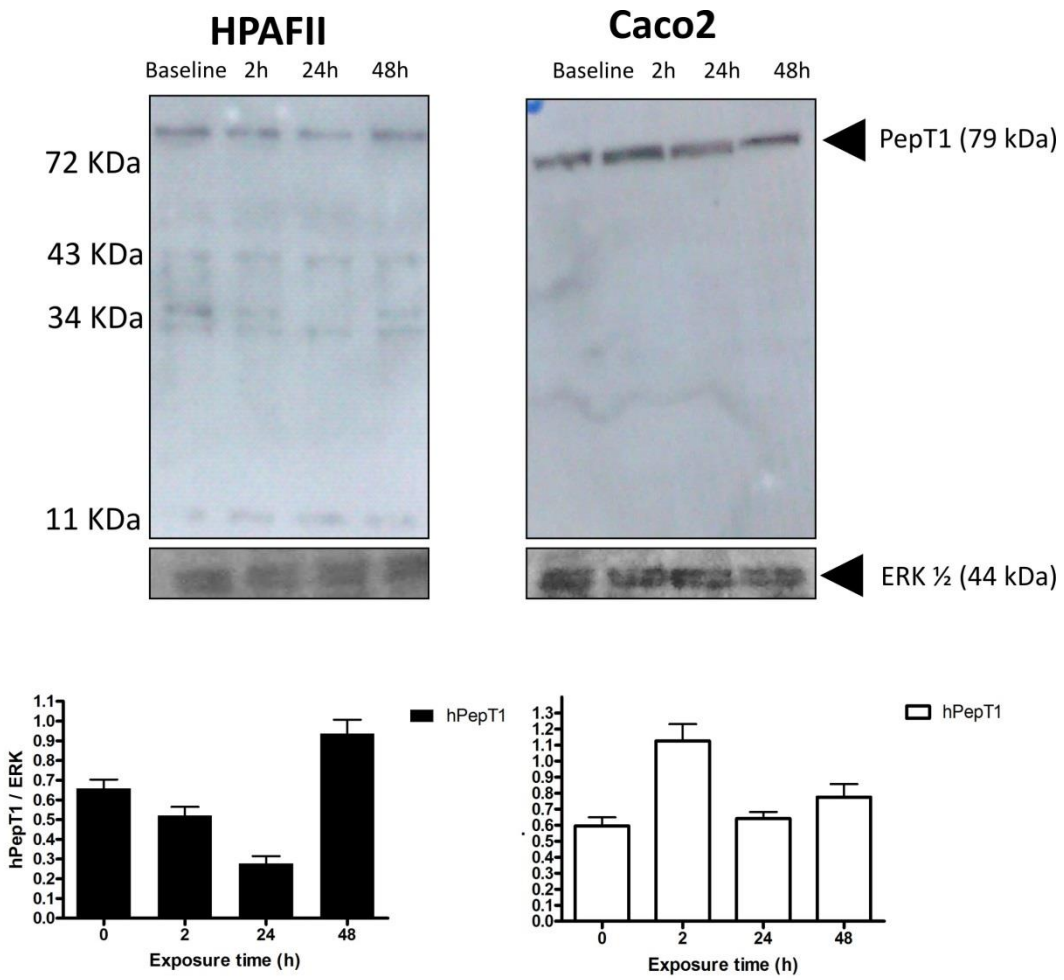


Figure 3.16 Change in PepT1 expression following cytokine treatment in HPAF and Caco-2 treatment. 50 ng/mL of IFN γ and 50 ng/mL of TNFα were added to the basolateral compartment and left incubating for 2, 24 and 48 h. Cells were then lysed for PepT1 expression levels. Data presented as mean (± SEM) of 3 independent experiments.

3.3.8 Cell viability and proliferation response after treatment with Ala(S)Asp-IB

Foley demonstrated the capacity of PepT1 to mediate the transport of thio-dipeptides and the use of this carrier transporter as a delivery approach of rationally designed drugs (Foley et al., 2009). Through an established collaboration with the Bailey Group at the University of Keele, we obtained a chimera compound composed of a peptide analogue

chemically coupled to the anti-inflammatory chimera compound, ibuprofen (Ala(S)Asp-IB). This material was tested for uptake into AsPc-1, as they express the oligopeptide transporter. PepT1 binding affinity for Ala(S)Asp-IB (Figure 3.17) has been confirmed through binding studies measuring the concentration at which this compound inhibited the uptake of radiolabelled D-Phe-L-Gln in *Xenopus laevis* oocytes expressing rabbit PepT1 (Foley et al., 2009).

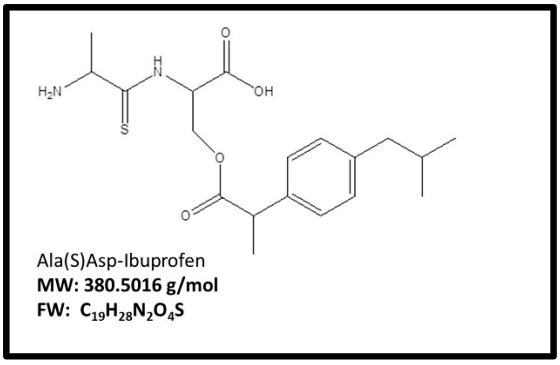


Figure 3.17 Ibuprofen modified chimera as a PepT1 substrate (Ala(S)Asp-IB).

NSAIDs, non-steroidal anti-inflammatory agents, have been previously reported to inhibit cancer cell proliferation (Hixson et al., 1994). To investigate if Ala(S)Asp-IB would affect cell proliferation through a PepT1-specific mechanism uptake, we assessed the actions of this chimera in PepT1 expressing and PepT1$^{-/-}$ cells (the same model as described in Figure 3.5). PepT1 expressing cells were found to be more susceptible to the anti-proliferative actions of Ala(S)-Asp-IB compared to PepT1$^{-/-}$ cells (Figure 3.18). At high concentrations (~200 and 500 μM) cell proliferation decreased in a comparable manner in both cell lines tested, possibly as a result of a toxicity threshold.

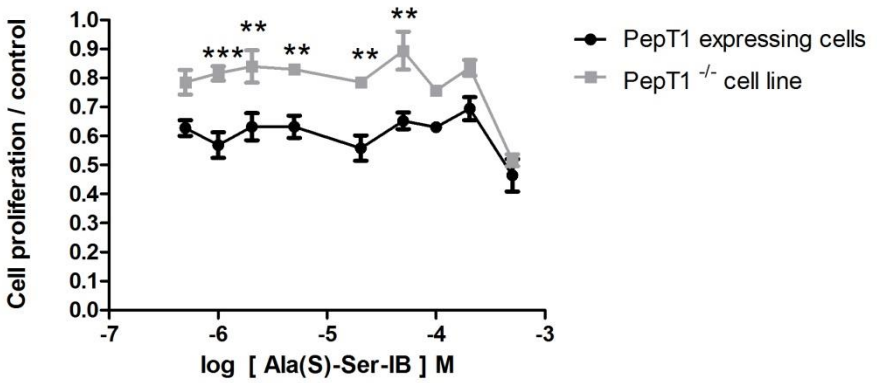


Figure 3.18 Ala(S)-Asp-IB affected differently cell proliferation rates observed in PepT1 expressing and PepT1$^{-/-}$ cells. AsPc-1 and AsPc-1 PepT1$^{-/-}$ cells were exposed to different concentrations of Ala(S)-Asp-IB during a 24 h period. Values are expressed as mean ± SEM from 3 independent experiments; **p< 0.01; ***p< 0.001.

Following this, we wanted to confirm the involvement of PepT1 in the uptake of chemically modified ibuprofen molecule achieved using the Ala(S)Asp-IB chimera. For this, PepT1 function was blocked through a specific substrate: Gly-Sar (Brandsch, 2009; Hu et al., 2008; Ocheltree et al., 2005; Thwaites et al., 1993). The rate of cell proliferation induced by Ala(S)Asp-IB was significantly decreased when compared to AsPc-1 cells cultured in media supplemented with Gly-Sar, suggesting the involvement of PepT1 in the uptake of Ala(S)-Asp-IB (Figure 3.19).

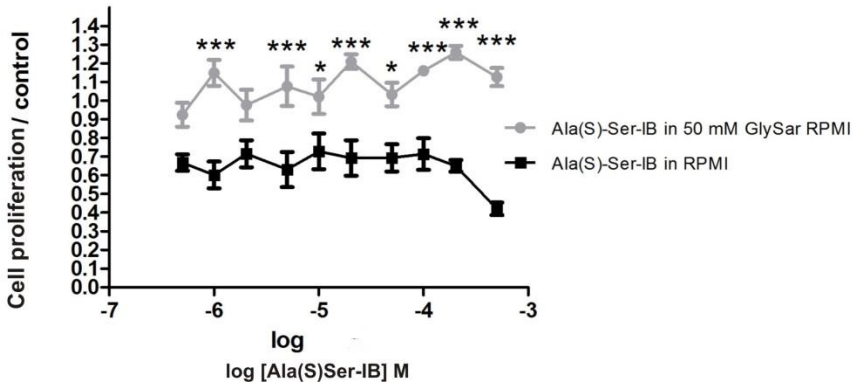


Figure 3.19 Blocking PepT1 function in AsPc-1 cells resulted in an increased proliferation level when compared to cells with normal PepT1 function after exposure to Ala(S)-Asp-IB. Proliferation rate of AsPc-1 cultured in normal media compared to AsPc-1 cells cultured in media supplemented with Gly-Sar after being exposed to Ala(S)-Asp-IB. Values are expressed as mean ± SEM from 3 independent experiments; * $p< 0.05$; *** $p< 0.001$.

Finally, to assess the actions of Ala(S)Asp-IB on cell viability, an MTT assay was carried out. Cell viability decreased significantly in WT AsPc-1 cells compared to PepT1$^{-/-}$ cells suggesting that the compound Ala(S)-Asp-IB could be taken up by the oligopeptide transporter, PepT1 (Figure 3.20-A).

The best characterized intracellular targets for anti-inflammatory drugs (NSAIDs) are inhibitors of the cyclooxygenase enzymes, Cox-1 and Cox-2 (Vane et al., 1998). Cox-1 is constitutively expressed in the majority of tissues and has a role in normal tissue homeostasis whereas Cox-2 is up-regulated as a stress response induced by inflammatory cytokines, growth factors and oncogenes (de Groot et al., 2007; Vane et al., 1998). Therefore, we investigated Cox-2 levels after cells were exposed to the PepT1 specific anti-inflammatory compound (Figure 3.20-B). No signal for Cox-2 expression was detected in PepT1 expressing cells followed by exposure to Ala(S)Asp-IB whereas in PepT1$^{-/-}$ a signal could be observed. This data suggested that the chemically modified ibuprofen was more

effective at reducing Cox-2 levels in PepT1 expressing cells compared to cells in which PepT1 expression was reduced.

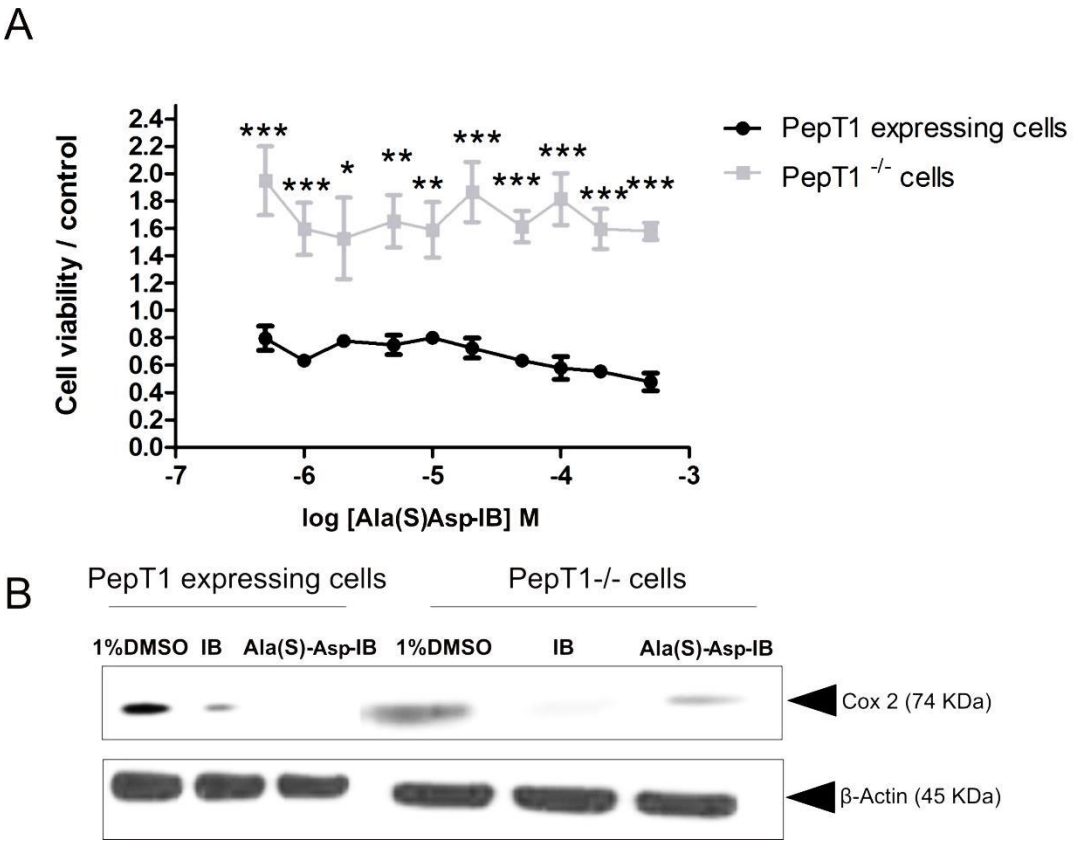


Figure 3.20 Cell viability response decreased in PepT1 expressing cells compared to PepT$^{-/-}$ cells; Cox-2 levels were absent in PepT1 expressing cells after Ala(S)-Asp-IB treatment. (A) Cell viability assay after AsPc-1 and AsPc-1 PepT1$^{-/-}$ cells were exposed to Ala(S)-Asp-IB during a 24 h period. Values are expressed as mean ± SEM from 3 independent experiments; * p<0.05; ** p< 0.01; *** p<0.001; **(B)** Cox-2 expression levels after AsPc-1 and AsPc-1 PepT1$^{-/-}$ cells were exposed to 100 μM of Ala(S)-Asp-IB and IB during a 24 h period.

3.4 Summary of results

- PepT1 is expressed at the cell surface of PDAC derived cell lines, whereas in non-cancerous pancreatic cell lines it localised predominantly at the cytosol and cell nuclei;
- PepT1 is expressed in PSC lysates from patients diagnosed with CP and PDAC;
- PepT1 immuno-labelling at the cell surface of malignant transformed pancreatic ducts is more prominent compared to morphologically normal ducts;
- PepT1 is expressed in tissue biopsies from patients diagnosed with CP and PDAC;
- BCH treatment decreases AsPc-1 cell viability through PepT1;
- Chemical coupling of gemcitabine to an hydrolysis resistant thio-dipeptide increases the specificity of the complex being taken by PepT1 as cell viability and proliferation decreases faster;
- Chemical coupling of gemcitabine to an hydrolysis resistant thio-dipeptide decreases cell viability more efficiently than the parent compound;
- PepT1 redistributes at the cell surface of inflamed pancreatic cells in later stages of CP;
- Chemical coupling of ibuprofen to an hydrolysis resistant thio-dipeptide decreases cell proliferation and viability and also affects Cox-2 expression levels.

3.5 Discussion

The use of specific drug transporters as molecular targets in cancer therapy has been considered for targeting tissues selectively as a means to reduce systemic toxicity. Amongst the wide range of membrane transporters, PepT1 is perhaps the drug transporter in the mucosal drug delivery field that has caught the most attention recently. As well as nutrients, some of these transporters are also capable of transporting anti-cancer drugs or anti-cancer agents in a pro-drug form. The robustness of PepT1 to adverse environmental conditions such as to the cytotoxic effects of conventional chemotherapy raises the possibility that PepT1 might pose as a good and robust target for drug delivery.

PepT1, normally restricted to the apical surface of small intestine where it functions to absorb di- and tri-peptides (Fei et al., 1994), has been reported to be expressed in cells derived from gastric cancer, osteosarcoma, bladder cancer, cholangiosarcoma and in PDAC (Gonzalez et al., 1998; Inoue et al., 2005; Knutter et al., 2002). In particular, Gonzalez´s work highlighted the overexpression of PepT1 in two PDAC cell line models with a ductal origin and a K-Ras genotype: AsPc-1 and Capan2 (Gonzalez et al., 1998). This work has reproduced those findings; PepT1 expression was confirmed in three PDAC derived cell lines, Capan2, HPAF II and AsPc-1 (Figure 3.1 - A). For localisation studies, cells were labelled with a polyclonal antibody raised against a peptide mapping near the C –terminus of PepT1 from human origin (Figure 3.1-B). Both PDAC cells showed a similar labelling pattern suggestive of both plasma membrane and intracellular vesicle distribution, whereas in the normal pancreatic cell line, labelling was restricted to the cytosol and cell nuclei (Figure 3.1-B).

Being a transporter system that works through a proton gradient, it was not unexpected to find PepT1 localised in intracellular vesicles. Nevertheless, these findings are consistent with Bockman´s observations proposing there is a considerable amount of PepT1 present in intracellular vesicles in a steady-state (Bockman et al., 1997). This could be down to the presence of small peptides resulting from incomplete protein breakdown that are then co-transferred from the lysosomes using the H^+ transport through the membrane (Bockman et al., 1997). Knowing that one of the main functions of lysosomes is to maintain a low internal physiological pH, such localisation could be facilitating the transport of oligopeptides present within these vesicles into the cytoplasm. In the same study, the authors co-localised PepT1 and Lamp-1, a lysosomal marker, in Caco 2 cells suggesting that at steady state PepT1 localizes in lysosomes. From the data obtained, it cannot be concluded exactly where these vesicles localised as a specific marker for lysosomal identification was not used. However, assuming that such vesicles were lysosomes, a possible physiological function for the localisation of PepT1 inside the lysosomes would be down to PepT1 function through an endocytic pathway, as suggested by Gonzalez (Gonzalez et al., 1998).

Next, we compared the expression levels between two cancer cell line models and the pancreatic ductal cell line derived from normal pancreatic ducts, HPDE. Both cancer cell lines, HPAFII and AsPc-1, showed significantly higher PepT1 expression levels compared to HPDE (Figure 3.1-C). As the challenge in the treatment of various epithelial cancers including PDAC, an intense desmoplastic reaction surrounding the organ contributes to limit access of drugs to these tumours, resulting in increased non-selective actions and off--target toxicity (Erkan et al., 2013; Feig et al., 2012). In PDAC, the intense stromal reaction is composed of PSCs, lymphatic and vascular endothelial cells, immune cells and fibroblasts (Bachem et al., 1998). All these elements create a complex microenvironment around the organ, promoting tumour growth, invasion, metastasis and ultimately resistance to

chemotherapy. PepT1 expression was examined in PSCs lysates from three patients diagnosed with PDAC and two with CP (Figure 3.2). A signal for PepT1 expression was confirmed in the five patients analysed. As PSCs are not from an epithelial origin, it was unexpected that PepT1 was expressed in myofibroblast-like cells. Thus, it remains to be confirmed if the oligopeptide transporter is functional or not in these particular stellate cells or if it serves a cellular function distinct from its ability to internalize small peptides. Nonetheless, this finding is consistent with the work carried out by Nakanishi, in which the fibroblastic cancer cell line HT1080 was reported to express the oligopeptide transporter at considerable levels (Nakanishi et al., 1997).

Established proton gradients at the cell surface of epithelial cells facilitate the uptake of substrates across oligopeptide transporters and through the polarised nature of cells, an acidic pH is maintained at the membrane surface (Mrsny, 1998). Mesenchymal cells, like PSCs, typically lose this polarised status, and cannot maintain a specific trans-epithelial pH gradient required to facilitate the uptake through oligopeptide transporters. A local injection of an anti-cancer agent capable of entering cells through an oligopetide transporter would circumvent unwanted uptake by PepT1 expressed in other organs, potentially improving its delivery to cancer cells. Additionally, the tumour microenvironment is known to be very acidic when compared to the normal tissue; such acidic conditions might favour the function of PepT1 which is activated by an inward proton gradient and require an acidic pH for optimal substrate uptake conditions (Figure 1.14). Supporting this, some PepT1 labelling was observed in the stroma of biopsy samples collected from cancer patients (Figure 3.3). Most, if not all, of the chemotherapeutic strategies to target PDAC are directed at the cancer and not the microenvironment. These findings have potentially highlighted a novel strategy of targeting the tumour stroma through the oligopeptide transporter in a way to increase the

tumour uptake. This idea is based upon a very limited patient sample size. Thus additional studies would be required to adequately test this possibility.

The majority of pancreatic carcinomas have a ductal origin (Von Hoff et al., 2005) and derive from the same gut tube that originates the small intestinal epithelia in which PepT1 is expressed as an important component of dietary nutrient absorption (Fei et al., 1994; Liang et al., 1995). It is possible to postulate that the gene expression imprinted in a specific cancer cell type, in this case from pancreatic origin, may be related to its embryological derivation. Cancer cells derived from tissue sharing an embryological origin in the primitive gut tube, such as the exocrine pancreas, may overexpress PepT1 preferentially over other transporter systems (Mrsny, 1998). After confirming PepT1 expression and localisation in cancer cell lines from PDAC origin (Figure 3.1), PepT1 distribution was also confirmed for the first time in pancreatic tissue biopsies derived from patients diagnosed with CP and PDAC (Figure 3.3). In particular, an increase in PepT1 localisation at the cell surface of cancerous pancreatic ducts could be observed compared to what was observed in non-cancerous biopsies (Figure 3.4). Despite the fact that the adult pancreas is a relatively quiescent organ, pancreatic ducts are structures with a very active role in the neogenesis of the pancreas (Bardeesy and DePinho, 2002). Since PepT1 was observed to be distributed at the basal and lateral surfaces of cells present in morphologically altered pancreatic ducts, it would be likely that the chances of targeting the organ more selectively through the blood stream would be considerably higher.

Cancer cells are metabolically more active than non-cancer cells and the high expression of PepT1 in some cancers has been shown to be related to an increased demand in nutrients required by fast growing cancer cells. Thus, inhibition of PepT1 might result in a novel strategy to delay or stop tumour growth. Manipulation of LATs activity has been speculated to have anti-cancer implications. Besides, due to the inhibition of LAT1 activity in tumour

cells was suggested to be effective in supressing tumour cell growth through essential amino acid deprivation in tumour cells (Kanai and Endou, 2001; Kim et al., 2002). Mitsuoka and co-workers synthesized a dipeptide, 4-(4-methoxyphenyl)-L-phenylalanyl sarcosine, able to inhibit PepT1 function; this resulted in the complete suppression of a xenograft model of human pancreatic AsPc-1 cells reported to express high levels of PepT1 (Gonzalez et al., 1998; Mitsuoka et al., 2010).

BCH has been described to inhibit LATs (Kim et al., 2008; Nawashiro et al., 2006). Mitsuoka observed the capacity of this inhibitor in inducing cell death by decreasing ATP content in AsPc-1 cells expressing PepT1 but failed to compare the outcome to a cell line with reduced or absent levels of PepT1 (Mitsuoka et al., 2010). To circumvent this, a cell line stably expressing reduced levels of PepT1 was created (Figure 3.5). Subsequently, differences in cell viability response to BCH between cells expressing PepT1 and PepT1$^{-/-}$ cells were investigated. After 48 h, PepT1 expressing cells presented a significant decrease in cell viability compared to PepT1$^{-/-}$ cells. These observations were consistent with the study carried out by Mitsuoka and suggested that inhibition of PepT1 might hinder AsPc-1 cell growth (Mitsuoka et al., 2010). Our data suggests that PepT1 might be a new target for suppression of pancreatic cancer cell growth (Figure 3.6).

The use of specific transporters as molecular targets in cancer therapy has revealed to be a promising strategy for tissue-selective drug delivery, while minimizing off-targeted toxicity. Pro-drugs can be designed to target specific antigens, enzymes or even peptide transporters. This can be achieved through conjugation of a specific tumour ligand to the chemotherapeutic drug via a cleavable linker (Han and Amidon, 2000). Coupling amino acids to anti-cancer agents to make them into PepT1 substrates represents an attractive method for targeted drug delivery. Through expression of mammalian PepT1 in *Xenopus*

laevis oocytes as a model system, essential residues in the functioning of PepT1 have been identified and a substrate template model has been established (Bailey et al., 2005; Bailey et al., 2006; Foley et al., 2009; Foley et al., 2010; Meredith, 2009). Through a collaboration established with the Bailey and Meredith group from the University of Keele, it was possible to chemically test PepT1 substrates generated from the chemical coupling of gemcitabine with a specific peptide in our *in vitro* model for PDAC (Figure 3.7).

Out of three possible chimeras, the most promising one in eliciting a reduction in cell proliferation and viability response was chosen and further characterised (Figure 3.8). The role of PepT1 involved in the uptake of gemcitabine chimera was assessed in PepT1$^{-/-}$ and PepT1 expressing cell lines. Chemically modified gemcitabine was revealed to have an enhanced cytotoxic effect in PepT1 expressing cell lines compared to cells in which the expression of the oligopeptide transporter was reduced. The calculated EC_{50} in the cell line expressing PepT1 was nearly fivefold lower (~11 μM) than in the PepT1$^{-/-}$ cell line (~54 μM) (Figure 3.9). In terms of proliferation, compound C decreased cell proliferation in a more pronounced manner in AsPc-1 cells when compared to cells with reduced levels of PepT1 expression (Figure 3.10).

After confirming that compound C decreased cell viability more efficiently than gemcitabine (Figure 3.11), competitive assays were used to examine the role of PepT1 in this outcome; cells treated simultaneously with compound C and a substrate for PepT1 (Gly-Sar) had greater level of viability compared to cells treated with compound C only. This suggested the potential involvement of Gly-Sar in blocking partially PepT1 uptake (Figure 3.12). Finally, the involvement of PepT1 in the uptake of gemcitabine chimeras was evaluated. Cell viability did not differ between PepT1 expressing and PepT1$^{-/-}$ cells after treatment with 10 mM BCH,

previously shown to affect cell viability through PepT1 inhibition. This data reinforced the role of PepT1 in the uptake of the gemcitabine chimera (Figure 3.13).

The correlation between the presence of inflammation and the development of pre-cancerous lesions at various anatomic sites is well established (Mantovani et al., 2008). Accumulating evidence has defined pre-existing and longstanding CP as a pre-empting factor for PDAC (Howes and Neoptolemos, 2002). CP and PDAC share some similarities and the most striking one is the abundant desmoplastic response (Logsdon et al., 2010). Several studies have reported PepT1 expression in inflamed cells (Buyse et al., 2002; Ingersoll et al., 2012; Vavricka et al., 2006; Wang et al., 2013; Wang et al., 2011), yet there are no reports about PepT1 distribution in inflamed pancreatic tissue. To address this, PepT1 distribution was assessed in five different patients diagnosed with CP (Figure 3.3 and Figure 3.14). PepT1 was observed to localize at the cell surface of inflamed ducts. Despite the controversy, for some, CP is still one of the biggest risk factors for PDAC (Howes and Neoptolemos, 2002); the detection of PepT1 in inflamed biopsy samples may provide both a diagnostic tool and a novel way to target inflamed pancreas prior to malignant transformation.

These findings support the idea that PepT1 might be used as a strategy to target anti-cancer agents as a way to increase selectivity and to minimize toxicity. In an attempt to develop an *in vitro* model of CP, epithelial-like HPAFII cells were allowed to polarise and then were exposed to two different cytokines (Figure 3.16). Due to lack of reproducibility, it was not possible to create an inflamed cell line model derived from pancreatic cancer (Figure 3.16).
Cyclooxygenase is an enzyme responsible for mediating the conversion of arachidonic acid to prostanoids, including prostaglandins, prostacyclin and thromboxane, which are mediators of acute and chronic inflammation (Vane et al., 1998). Cox-2 plays an important

role in inflammation and the increased production of prostaglandins has been associated with tumourigenesis (Dang et al., 2002). Some studies have demonstrated that NSAIDs had a beneficial effect in the prevention and treatment of colon cancer and ovarian cancer (Kune et al., 2007; Rodriguez-Burford et al., 2002). Andrews and co-workers reported that ibuprofen was able to significantly supress cell proliferation in a prostate cancer cell line at clinically relevant concentrations (Andrews et al., 2002). So far, the use of NSAIDs for cancer prevention and therapy remains controversial as many clinical studies have reported the long-term side effects ranging from GI toxicity to cardiotoxicity; as a result many Cox-2 specific agents have been withdrawn from the market (Rayburn et al., 2009).

Through collaborative work, ibuprofen was chemically modified and attached to a thio-dipeptide pro-drug and shown to be stable and resistant to hydrolysis (Foley et al., 2009)(Figure 3.17). Firstly, the impact of Ala(S)Asp-IB in reducing cell proliferation was assessed in PepT1 expressing and in PepT1$^{-/-}$ cells. Chemically modified ibuprofen into PepT1 substrates had a more pronounced effect on cell proliferation and viability in PepT1 expressing cells compared to control cells (Figure 3.18 and Figure 3.20-A). These findings are consistent with the recent study carried out by Lichtenberger, in which ibuprofen demonstrated a direct inhibitory effect on the growth of colon cancer cells (Lichtenberger et al., 2014). Likewise, our findings are in line with the effect reported by other NSAIDs drugs in reducing cancer cell proliferation (Hanif et al., 1996; Rodriguez-Burford et al., 2002). To assess the involvement of PepT1 in the uptake of Ala(S)Asp-IB, cells that were cultured in media supplemented with Gly-Sar showed a much higher proliferative rate compared to cells cultured in normal media; This suggests a blocking effect by Gly-Sar in preventing the compound uptake (Figure 3.19).

PepT1 as a Drug Delivery Target to Treat Chronic Pancreatitis and Pancreatic cancer

Cox-2 was reported to be expressed in patients with CP and PDAC and its pharmacological inhibition has been associated with an increased apoptotic rate in PDAC cells (Schlosser et al., 2002). This prompted us to differentiate Cox-2 levels in PepT1 expressing and PepT1$^{-/-}$ cells after exposure to Ala(S)-Asp-IB. A signal for Cox-2 expression was not detected in PepT1 expressing cells treated with Ala(S)-Asp-IB, suggesting the specific uptake and targeting of the chemically modified chimera. Cox-2 levels were diminished compared to the control, ibuprofen, in PepT1 expressing cells but not in PepT1$^{-/-}$ cells (Figure 3.20).

Targeting cancer cells with novel pro-drug chemotherapeutics functionally directed to nutrient uptake pathways has shown some promise in reducing side effects and increasing clinical efficiency (Cravo and Mrsny, 2013; Nakanishi, 2007) (Figure 3.21). Nonetheless, there are some pitfalls that need to be taken into account. Firstly, there are challenges in relying on membrane transporters as drug delivery targets; susceptibility to drug-drug and drug-endogenous ligand interaction, as well as sensitivity to changes in the microenvironment of the transporter as it is likely to occur in inflammation or infection, are two important hurdles that need to be considered (Lee, 2000).

It is becoming clearer that even though some cancer cells overexpress certain cellular markers, it is still not known if these receptors are functional or not. PepT1 up-regulation could be the result of a clonal expansion from the cell line when cultured *in vitro*, instead of representing the original tumour conditions (Mrsny, 1998). Secondly, the RNA message for PepT1 has been reported in the small intestine, liver and kidneys, albeit the primary site for PepT1 expression is the intestine (Fei et al., 1994; Liang et al., 1995). The uptake of anti-cancer compounds using PepT1 could therefore have the potential for secondary toxicity in other organs where the protein is expressed. With this is mind, it could be useful to understand what factors drive such up regulation in cancer cells. Finally, because there are

specific transcription factors that induce the expression of these transporter systems in cancer cells, it is not known if the same drivers are also up-regulating efflux pathways, such as P-gp (P-glycoprotein). Needless to say, an up-regulated efflux system could limit the effectiveness of anti-cancer compounds delivered to these cancer cells.

Important to this discussion are potential differences concerning the gene expression profile between cells cultured *in vitro* and the *in vivo* condition. Many of these differences may be the consequence of either tumour microenvironment or stromal gene expression that well characterizes PDAC. As future work, to confirm the specificity of PepT1 in taking up peptide modified chimeras such as gemcitabine and ibuprofen, the use of PepT1 null mice would be a very valuable tool to prove the concept of specific drug delivery through PepT1.

The importance of drug transporters in cancer biology and chemotherapy highlights the pivotal role of these molecules in the redesign of modern and targeted therapies. It could even be said that an exciting era to use these proteins as a cancer therapy tool has already begun. A more rational and sensible use of these molecules with unique patterns of expression will certainly underline the optimization of individualized cancer medicines. A rational drug design in combination with a rational drug delivery are factors that assume utmost importance and need to be integrated as major pillars sustaining an effective and promising cancer therapy. Until now, there is no known human pathology associated with the abnormal function of peptide transporters and no polymorphisms in human peptide transporters have been shown either. Understanding the mechanisms underlying the regulation of PepT1 will certainly be a key factor for the prediction of intra and inter individual variability of the compounds bioavailability, maximizing their efficacy ultimately.

PepT1 as a Drug Delivery Target to Treat Chronic Pancreatitis and Pancreatic cancer

Here, it was demonstrated that PepT1 is up-regulated in cancer cell lines compared to normal cells and in inflamed and malignant pancreatic human biopsies compared to normal tissue. This work suggested that a differential expression of transporters in cancers can be exploited for drug delivery or even used as diagnostic markers in the treatment or prevention of PDAC. In order to optimize efficacy versus safety outcomes, further work will need to be carried out to fully address this concept.

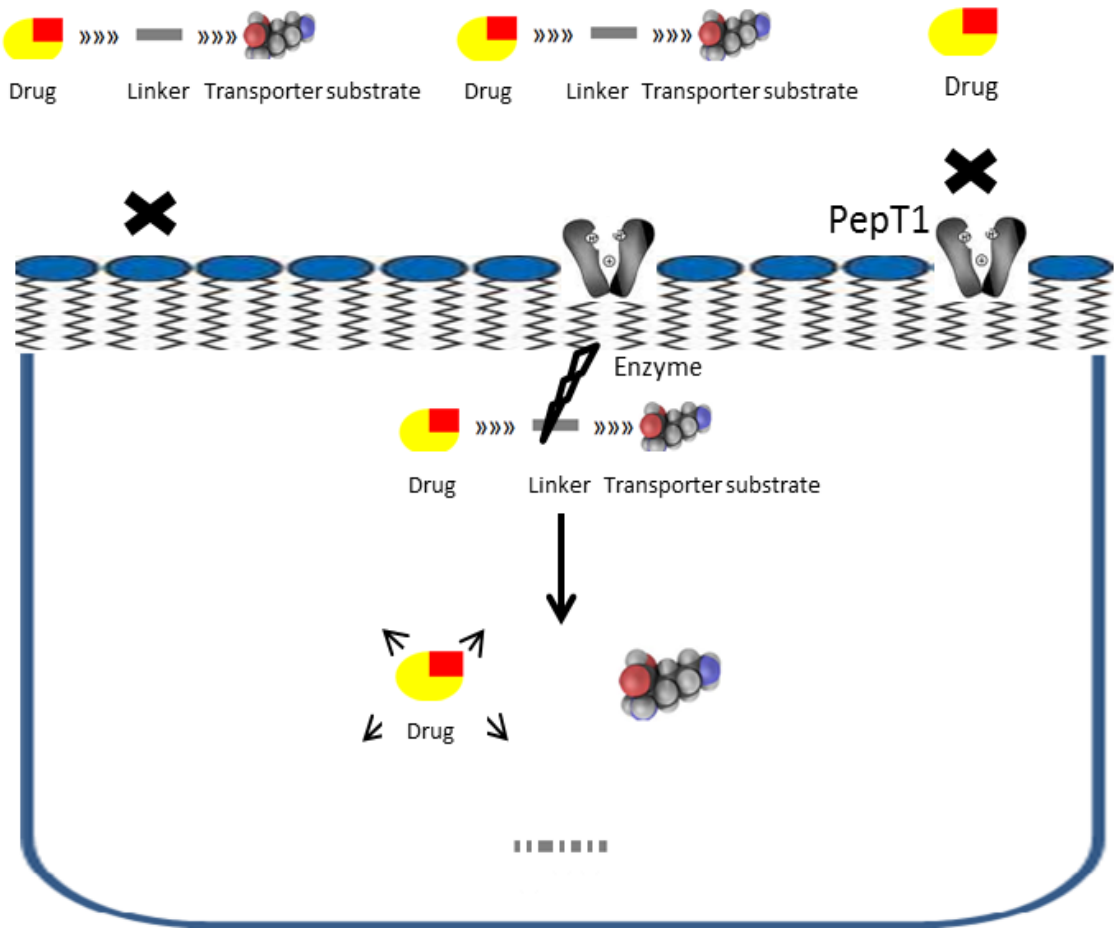


Figure 3.21 Diagram representing the pro-drug uptake model through PepT1 transporter (Cravo and Mrsny, 2013).

Ocln interactions with Hippo Pathway Signaling

Chapter 4

4 Ocln interactions with Hippo Pathway Signaling

4.1 Background

As part of their differentiation, epithelial cells polarise into apical and basal domains following their adjacent cell-cell contact. This differentiated organization functions to restrict lateral expansion, which is reflected in cell size, shape and proliferative properties. Differentiated cells have an apical-basal polarity defined by the apical membrane facing the outside surface of the body, or the lumen of internal cavities, and the basolateral membrane oriented away from the lumen (St Johnston and Ahringer, 2010). Lateral membrane interactions formed by AJs and the generation of apical-basal polarity appear to precede the initiation of TJ formation (Sawada, 2013). TJ complexes are composed of a branching network of independent sealing strands (Gonzalez-Mariscal et al., 2003). Each strand is formed by transmembrane proteins embedded in both plasma membranes and the extracellular domains interacting in homotypic and heterotypic contacts with TJ proteins of the adjacent cell (Tsukita et al., 2001). The establishment of TJ at the uppermost portion of the plasma membrane is the result of a polarised insertion of proteins; apical to basolateral polarity is required for restriction of plasma membrane components and for limiting the solute flux between adjacent cells. Three main conserved protein complexes are involved in the maintenance and establishment of the apical-basal polarity: Crumbs, PAR and Scribble (Benton and St Johnston, 2003; Bilder and Perrimon, 2000; Ebnet et al., 2001).

Fence and barrier function are two well-established properties of TJ (Rescigno et al., 2001); the other concerns the involvement of TJ molecules in the regulation of signaling transduction pathways (Balda and Matter, 2009). Of particular interest to this work is the possible TJ role as a platform that integrates Hpo pathway phosphorylation events. *YAP* and *TAZ* gene mutations have been demonstrated in several human epithelial cancers and YAP/TAZ over-expression in mammalian cells can trigger a plethora of oncogenic events (Sudol et al., 2012). Disrupted regulation of Hpo pathway elements, in particular YAP,

correlates with loss of epithelial cell crowding control leading to deregulated proliferation, a hallmark of epithelial cancers (Pan, 2010; Poon et al., 2011). Mutation at the phosphorylation site Ser^{127} in the *YAP* gene serves as a signal for the translocation of YAP to the nucleus where it binds to a transcription factor element, TEAD, which is responsible to drive the transcription of genes involved in promoting growth promoting and anti-apoptotic programs (Zhao et al., 2008). Thus, YAP/TAZ actions provide a means for individual cells within an epithelial sheet to monitor their cell density and polarity status.

A critical aspect of assuring reproducible cell density and polarity conditions requires mechanisms to sense the status of individual cells in the context of the epithelial sheet. Interactions between Hpo pathway elements and components of the APC and the AJ have been reported (Rajasekaran et al., 2004). Yet, E-cadherin localised at the AJ has been the only cell-cell contact sensor shown to be involved in the regulation of YAP and suppressing epithelial cell proliferation at high lateral cell density (Schlegelmilch et al., 2011). Therefore, it is reasonable to hypothesise that a TJ element would be involved in a feedback sensor mechanism to coordinate the completion of apical-basal polarity program with the suppression of cell proliferation coordinated by Hpo signaling. Indeed, it has already been hypothesised as an extracellular sensor able to detect competent TJs (Nusrat et al., 2005).

The aim of this chapter is to examine a possible link between the TJ protein Ocln and elements of the Hpo pathway that could provide a novel mechanism to control epithelial cell polarization in pancreatic cancer cells. Besides, this chapter also aims at analyzing the distribution of Hpo pathway elements in CP and PDAC. Finally, this work also explored if dobutamine, previously shown to induce YAP nuclear translocation, can affect Ocln expression and function (Cravo, et al., 2014, submitted). To address this, an epithelial cell line able to undergo apical-basal polarization (HPAFII) and another cell line that cannot (AsPc-1) were studied.

4.2 Materials and methods

4.2.1 Immunohistochemistry

Histological slides from fifteen human biopsies from five different patients (five from normal tissue, five from patients with CP and five from patients diagnosed with PDAC) were analysed for Ocln, YAP, TEAD and c-Yes distribution levels. Paraffin tissue sections were prepared according to section 2.10, Chapter 2. For antigen detection, slides were subjected to an overnight incubation in a humid chamber at 4 °C with antibodies recognizing Ocln, YAP, TEAD and c-Yes (Table 2.6 , Chapter 2) diluted 1:100. The control antibody used was rabbit antisera (Table 2.6, Chapter 2). After slides were mounted on DePex, they were allowed approximately 12 h to dry out; subsequently slides were examined as described in section 2.10, Chapter 2.

4.2.2 Immunostaining

AsPc-1 (passage 14), and HPAFII (passage 10) cells were grown on coverslips reaching up to 50 % confluency (generally 2 days for AsPc-1 and 5 days for HPAFII cells). For co-localisation experiments, after cells were fixed and permeabilised as described in section 2.9, Chapter 2, they were subjected to 1 h incubation exposed to simultaneously YAP and c-Yes, Ocln and c-Yes, Ocln and YAP and finally Ocln and TEAD (Table 2.6, Chapter 2) at 4 °C. Afterwards cells were washed and incubated with the appropriate secondary antibody at a 1:400 dilution (Table 2.6, Chapter 2). Subsequently, slides were mounted and analysed through confocal microscopy as described in section 2.9, Chapter 2.

4.2.3 Immunoprecipitation and Western Blot

For immunoprecipitation assay, AsPc-1 and HPAFII, passages 17 and 14 respectively, were lysed as described in section 2.4, Chapter 2. Whole cell lysates containing approximately 500 µg of protein were incubated with a pull-down antibody, YAP, (Table 2.6, Chapter 2) overnight at 4°C. Proteins were then isolated and analysed through Western Blot as outlined in section 2.6, Chapter 2. Five PSC lysates, obtained from Dr. Mert Erkan from The Technical University of Munich, were collected for YAP protein expression levels (three samples were isolated from patients diagnosed with PDAC and two from patients with CP). Lysates were then subject to Western Blot following the protocol outlined in section 5.1, Chapter 2.

4.2.4 Cell culture, and TER measurements

AsPc-1 (passage 9) and HPAF II (passage 8) cell lines were obtained from ATCC© and cultured as described in section 2.1, Chapter 2. TER measurements were carried out daily and performed as mentioned in section 2, Chapter 2.

4.3 Results

4.3.1 Ocln expression decreases and YAP expression increases with pancreatic cancer progression

TJs provide a coherent cell-cell adhesion structure acting as a barrier to the initiation and progression of cancer (Brennan et al., 2010). A hallmark of aggressive epithelial cancers, such as those derived from pancreatic acini and ducts, is loss of functional cell-cell contacts

(Hezel et al., 2006). The distribution of the TJ marker, Ocln, was assessed in inflammation and pancreatic cancer (Figure 4.1 – top panel and Figure 4.2). Ocln expression in non-inflamed and in non-cancerous pancreatic tissue, localised essentially at the cytoplasm and at the apical surface of ducts. In PDAC tissue, however, Ocln staining appeared to be substantial but did not distribute at the plasma membrane (Figure 4.2).

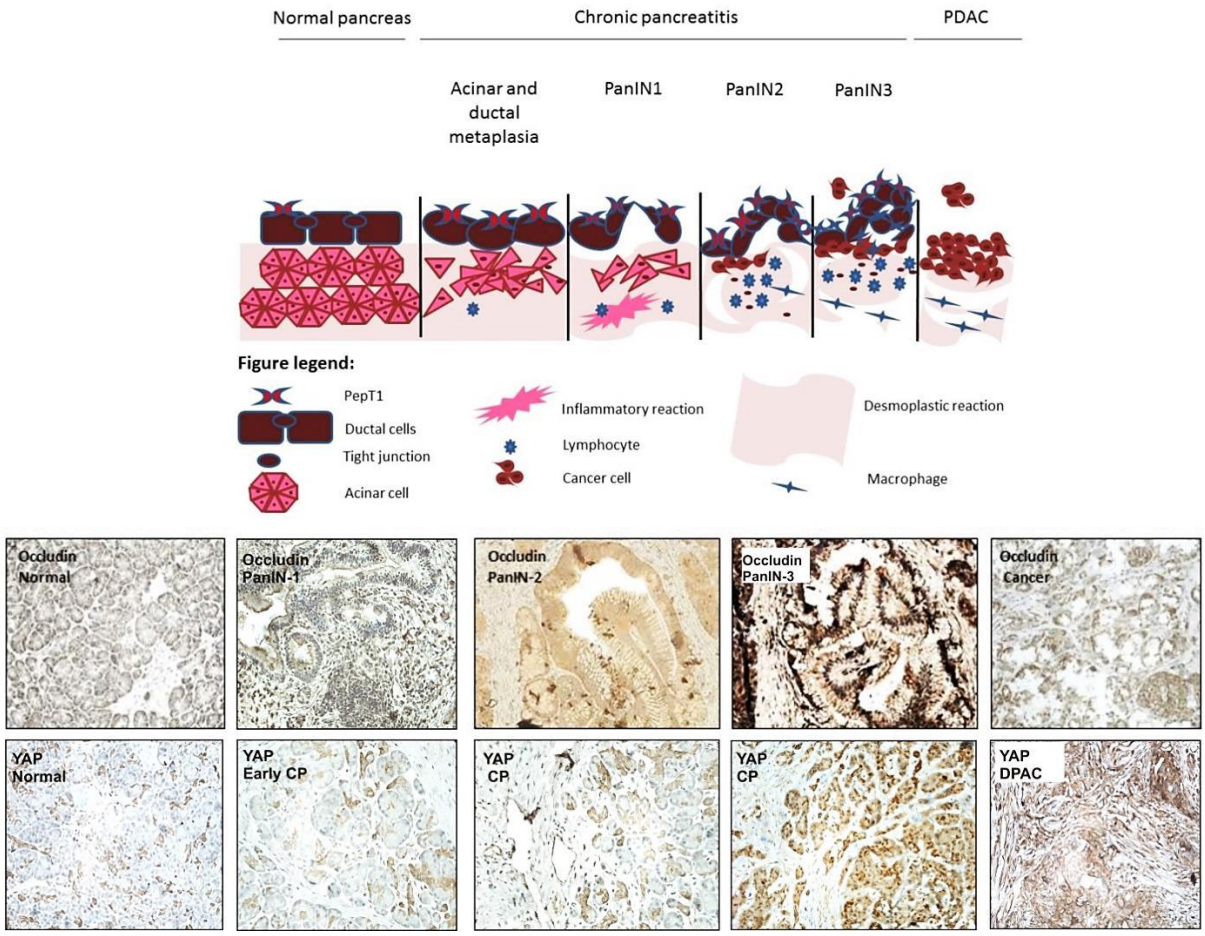


Figure 4.1 Ocln distribution in five histologically defined tissues representing the various stages of progression from normal pancreas through CP and PDAC. Weak to moderate Ocln staining intensity and moderate to strong YAP staining intensity was observed in PDAC biopsies.

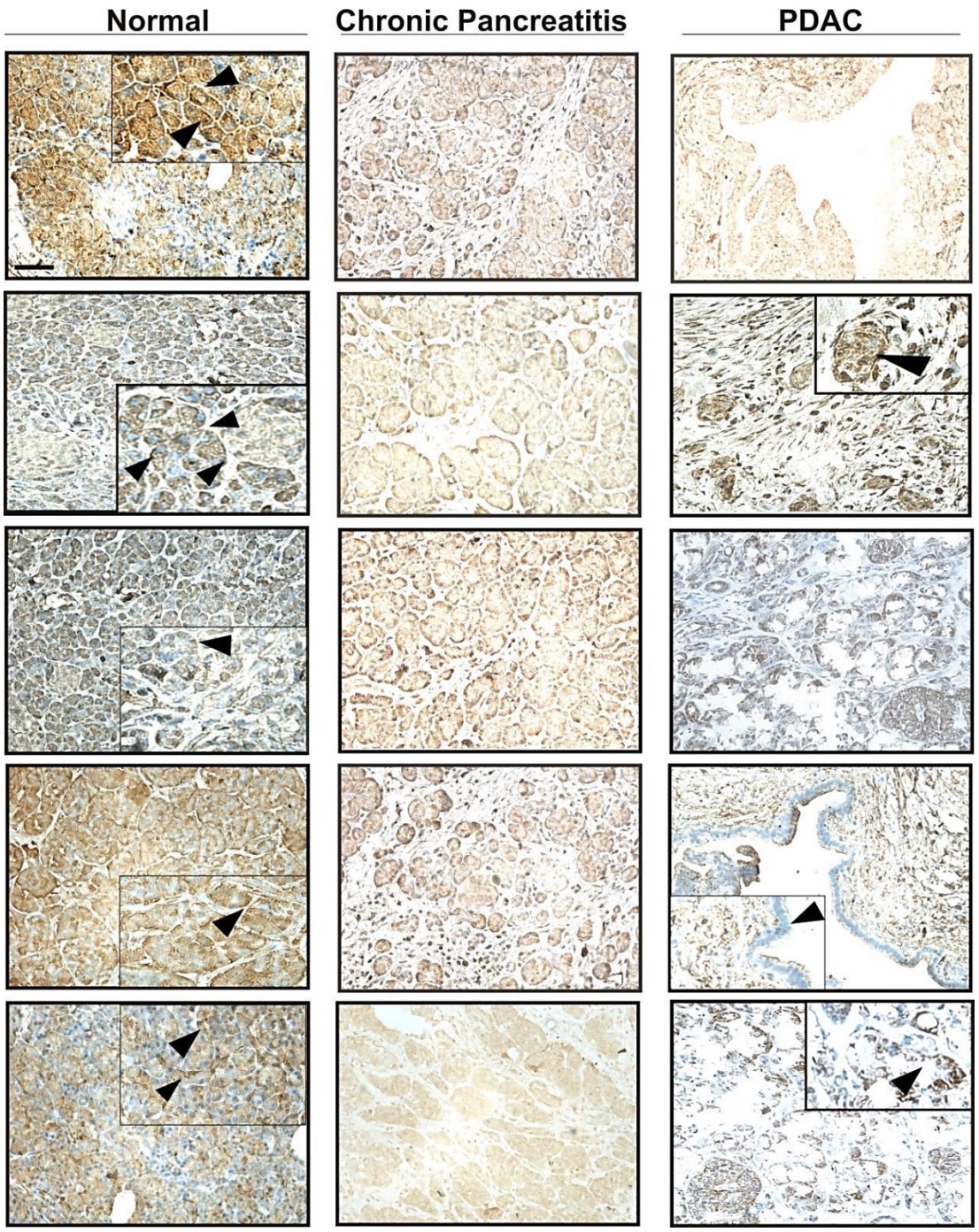


Figure 4.2 Ocln distribution decreases progressively in human pancreatic inflammation and cancer; Immunohistochemistry analysis of 15 human pancreatic biopsies (5 patients with normal pancreas, 5 patients with CP and 5 patients with PDAC) immunostained for Ocln; Insets with higher magnification of specific tissue regions are presented and black arrows are pointing at specific immuno-labelling within the tissue section; scale bar, 50 µm.

OcIn interactions with Hippo Pathway Signaling

The Hpo tumour suppressor pathway regulates cell proliferation and apoptosis in the context of establishing and maintaining proper lateral (planar) cell polarity that defines cell density within an epithelium (Martin-Belmonte and Perez-Moreno, 2012). YAP interacts with specific transcription factors to promote cell proliferation, organ growth and survival (Shao et al., 2014a). Disrupted regulation of Hpo pathway elements, in particular YAP, correlates with epithelial cell transformation (Pan, 2010; Poon et al., 2011). Since YAP was observed to be continuously overexpressed in inflammation (Figure 4.1 – bottom panel) and also in PDAC biopsy samples, YAP distribution from five different patients was analysed (Figure 4.3). YAP expression in normal pancreatic epithelial cells was detected mostly in the cytoplasm of acinar and ductal cells with minimal nuclear staining observed whereas in inflammation and more markedly in cancer, a strong nuclear signal could be observed. In cancer biopsies, strong YAP staining was detected in PanIN lesions; YAP was predominantly distributed in the nucleus, however some cytoplasmic expression could be observed in both inflamed and cancerous conditions.

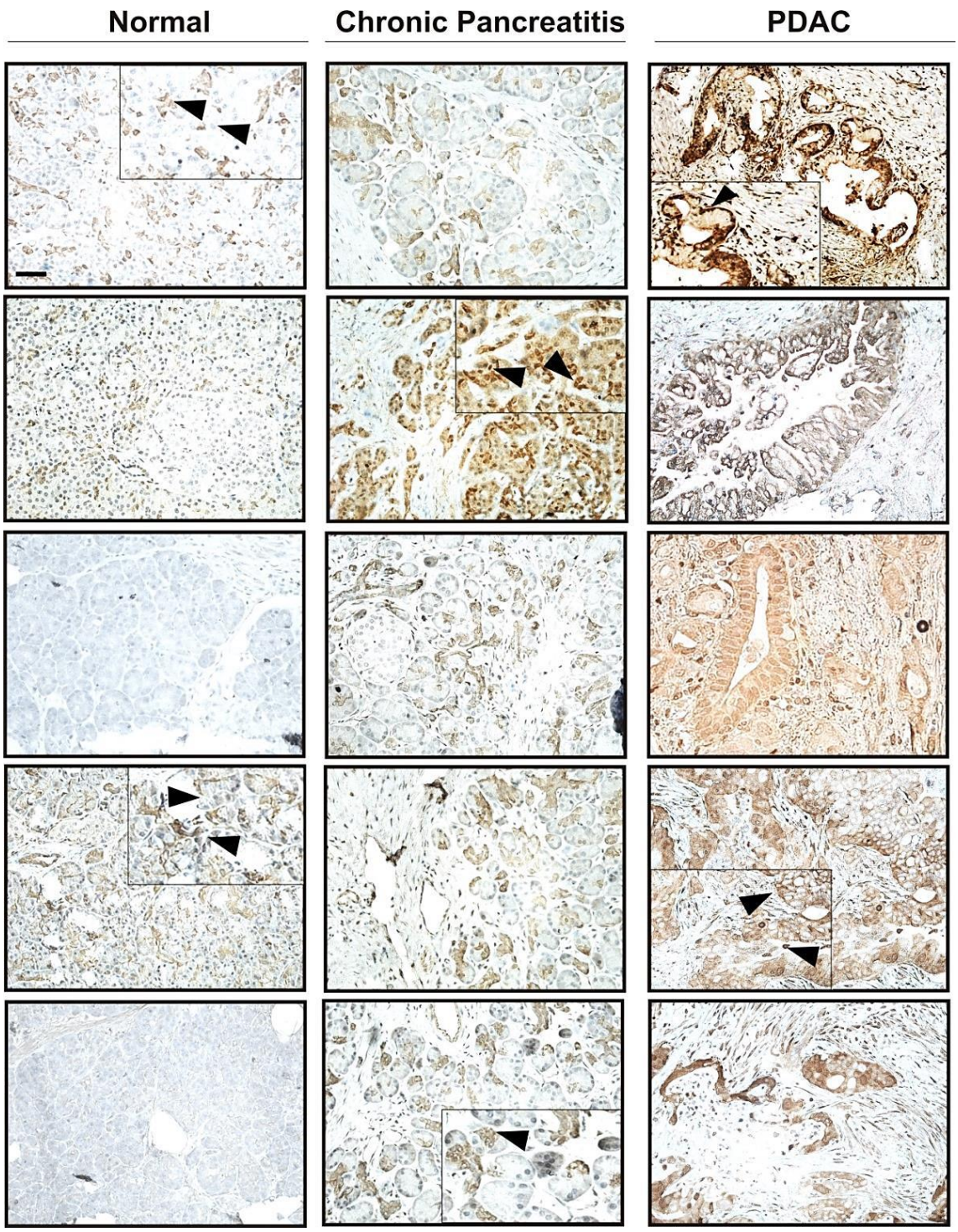


Figure 4.3 YAP distribution increases progressively from human pancreatic inflammation to pancreatic cancer; Immunohistochemistry analysis of 15 human pancreatic biopsies (5 patients with normal pancreas, 5 patients with CP and 5 patients with PDAC) immunostained for YAP; Insets with higher magnification of specific tissue regions are presented and black arrows are pointing at specific immuno-labelling; scale bar, 50 μm.

PSC take an important role in controlling the tumour microenvironment in pancreatic cancer (Omary et al., 2007), yet there are no studies reporting YAP activity in these cells. We sought to analyze YAP expression levels and correlated it with data obtained from the biopsy samples. YAP was detected in at least two patients diagnosed with PDAC and absent levels were observed in pancreatic inflammation (Figure 4.4). The results obtained from patients diagnosed with CP were not consistent with the biopsy samples analysed as a weak to moderate staining was obtained for the five patients diagnosed with CP. However, due to this limited set of patients, no further conclusions can be drawn.

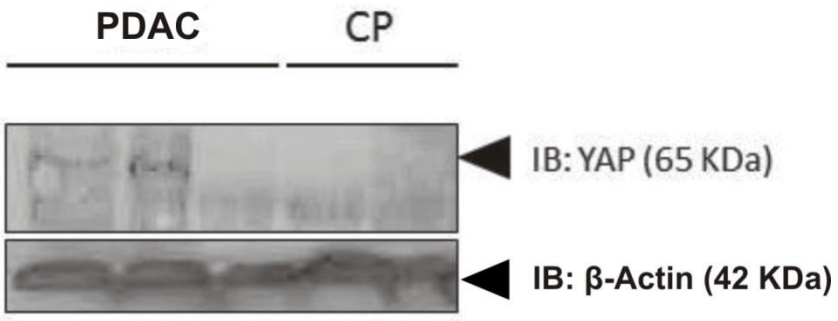


Figure 4.4 YAP expression was detected in PSC lysates from two patients diagnosed with PDAC but not in patients with CP. Immunoblot showing YAP levels in PSC lysates collected from patients diagnosed with either PDAC or CP.

4.3.2 Nuclear TEAD expression increases in PDAC

The Hpo signaling cascade regulates planar cell polarity and, in turn, controls cell proliferation and survival pathways (Pan, 2010). Translocation of YAP from the nucleus to the cytoplasm with its subsequent phosphorylation and degradation is correlated with suppression of cell proliferation (Pan, 2010). YAP does not contain an intrinsic DNA-binding domain but instead regulates target genes by interacting with DNA-binding transcription factors in the nucleus (Yu and Guan, 2013). One of these transcription factors is the

TEA-domain family member TEAD, regarded as the most potent targets of YAP, which functions to activate cell proliferation processes (Bao et al., 2011; Vassilev et al., 2001). To investigate TEAD tissue distribution and localisation, human pancreatic biopsies from five different patients diagnosed with either CP or PDAC were analysed and compared to normal tissue. TEAD labelling in normal pancreas was restricted to the cytoplasm and very minor amounts could be observed in the nucleus. In CP, TEAD signal was stronger compared to non-inflamed and non-cancerous biopsies. PDAC tissue showed some cytoplasmic staining but more strikingly an extensive nuclear localisation (Figure 4.5).

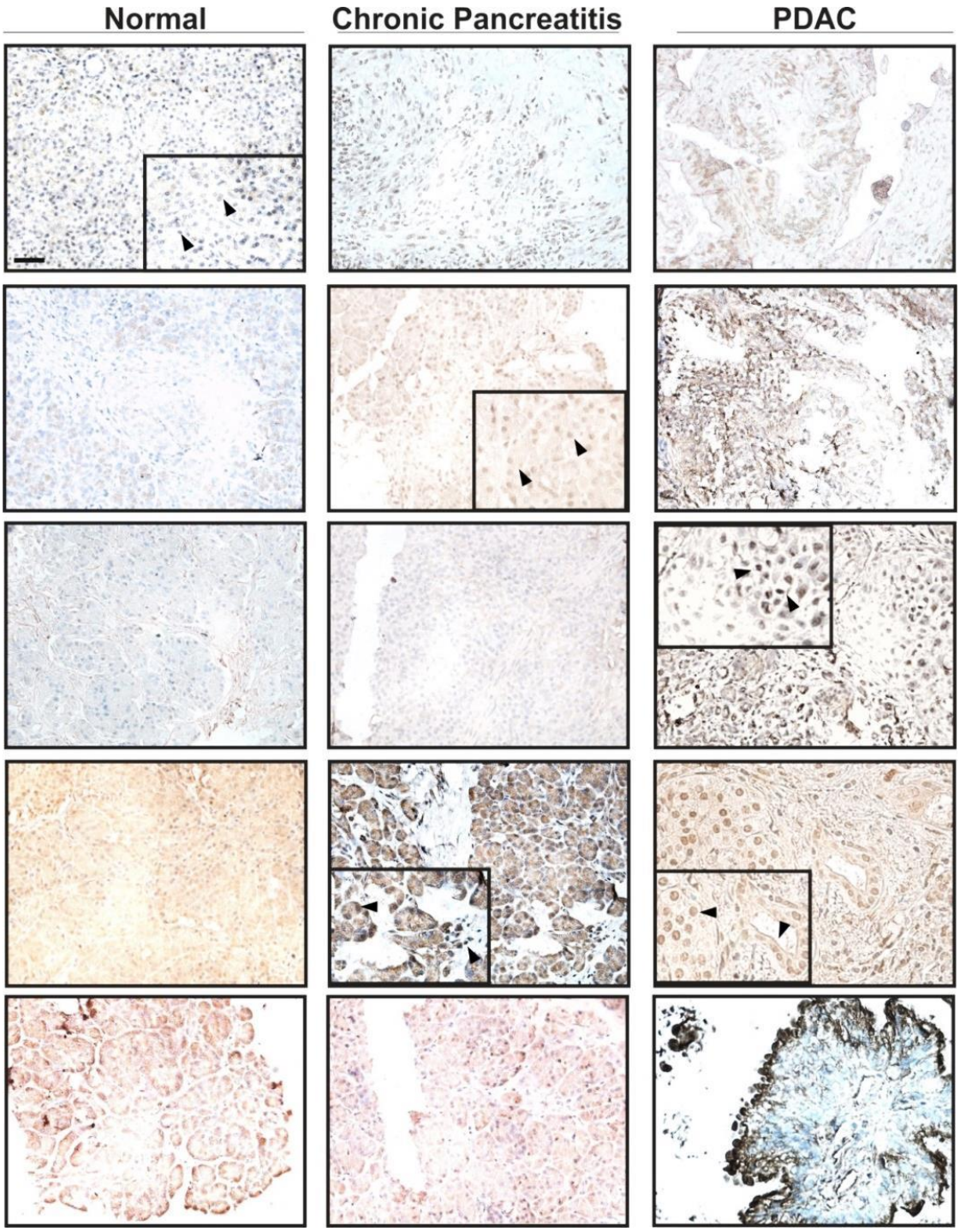


Figure 4.5 Nuclear TEAD distribution increases progressively in human pancreatic inflammation and cancer; Immunohistochemistry analysis of 15 human pancreatic biopsies (5 patients with normal pancreas, 5 patients with CP and 5 patients with PDAC) immunostained for TEAD; Insets with higher magnification of specific tissue regions are presented and black arrows are pointing at specific immuno-labelling; scale bar, 50 µm.

4.3.3 Ocln co-localises with Hpo pathway elements in HPAFII and AsPc-1 cells grown at low density

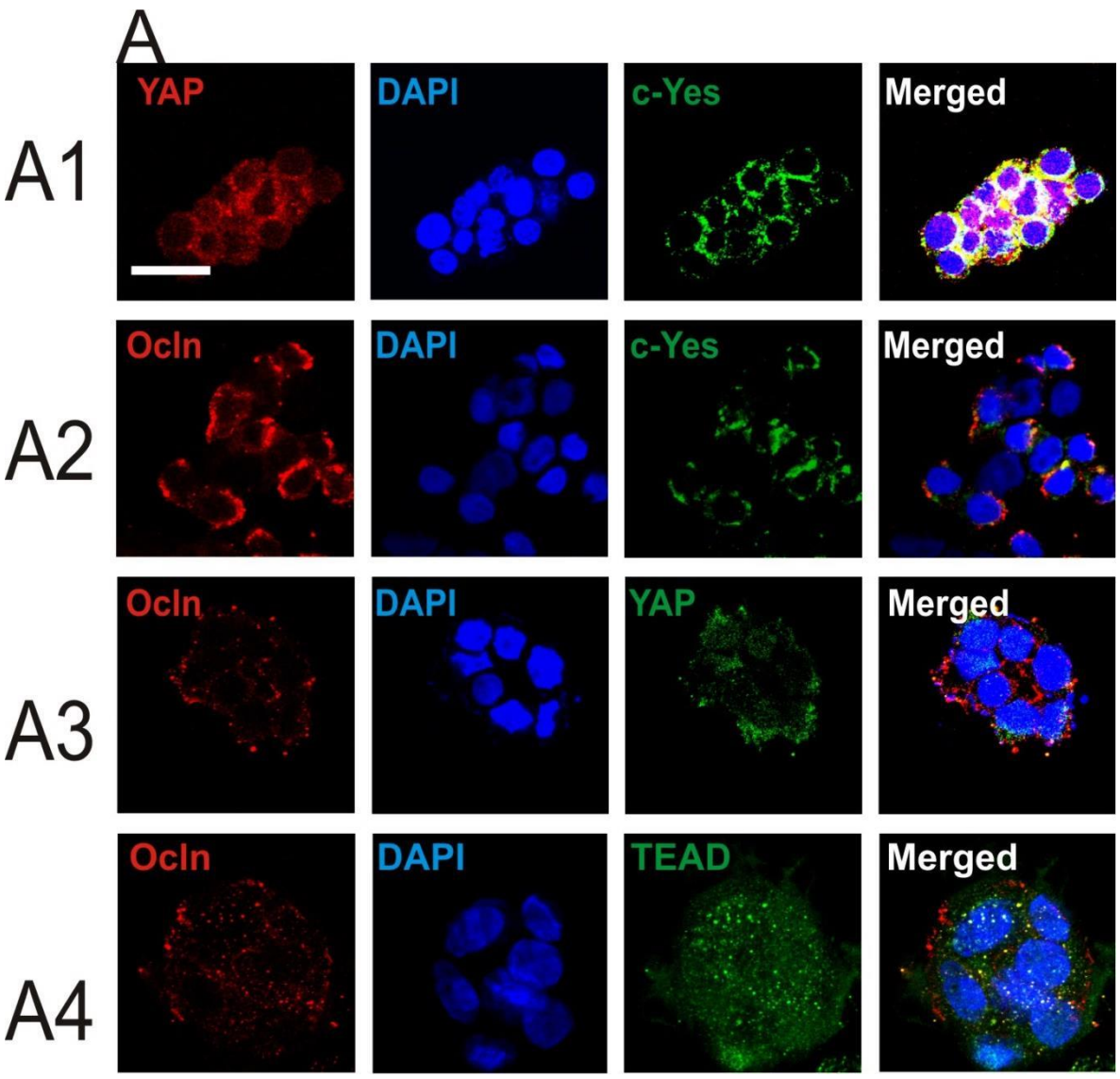
Despite the fact that Ocln was the first integral membrane protein identified (Furuse et al., 1993) acting in the organization and dynamic function of the tight junction, recent studies have been showing that it does not have a critical role in barrier function; mice lacking Ocln can produce an efficient barrier function through claudin-based TJs. Still, the Ocln knockout mice have a peculiar phenotype characterized by bone thinning, brain calcifications, stunted growth, and others (Saitou et al., 2000). Past studies have shown that down-regulation and epigenetic silencing of Ocln correlates with cancer progression (Osanai et al., 2006; Tobioka et al., 2004a; Tobioka et al., 2004b). In addition, loss of Ocln expression or disruption has been observed to correlate with epithelial cell transformation (Sawada, 2013). The coiled coil domain of Ocln interacts selectively with c-Yes and interactions between c-Yes and Ocln appear essential for TJ formation (Chen et al., 2002b; Nusrat et al., 2000). As c-Yes is a *bona fide* binding partner of YAP which in turn plays a critical role in Hpo signaling and associates with the cytoskeleton organized at TJ structures (Aragona et al., 2013), we conjectured that this integral plasma membrane protein involved in cell-cell contacts could function as a potential sensor to coordinate lateral (planar) and apical-basal epithelial cell polarity with proliferation control through the Hpo pathway.

In humans, the phenotype for mutations in Ocln is restricted to the brain, again suggesting some functional redundancy for Ocln. Thus, essential Ocln function(s) imparting these phenotypes would seem to involve cell processes beyond the establishment of the epithelial barrier. Important to this discussion is the fact that $Ocln^{-/-}$ mice exhibit chronic inflammation and hyperplasia of their gastric epithelium, indicating that Ocln is involved in epithelial differentiation (Furuse, 2009). Epithelial inflammation is associated with reduced barrier function and the internalization of TJ proteins, such as Ocln, although it has minimal effects

on the cytoplasmic plaque proteins and cytoskeleton associated with TJ structures (Bruewer et al., 2003). Importantly, YAP-mediated Hpo signaling can occur through the cytoskeleton (Aragona et al., 2013) and epithelial cancers involving dysfunctional Hpo pathway elements have been identified (Pan, 2010).

While the location of polarity elements and many of their interactions have been established for polarised epithelia, the identification of Ocln as a sensor for Hpo pathway signaling provides an important link for TJ signaling to control the balance between epithelial cell proliferation versus polarization. We first characterized potential protein-protein associations in HPAFII, capable of undergoing apical-basal polarization, grown on plastic as small clusters where nascent and lateral cell-cell contacts had been established. Through confocal analysis and specialised co-localisation software, we examined the interactions between YAP and c-Yes, Ocln and c-Yes, Ocln and YAP and finally Ocln and TEAD in HPAFII and AsPc-1. YAP and c-Yes, previously shown to interact (Sudol, 1994), were observed to co-localise extensively at the cell membrane and cytoplasm but not in the nucleus of HPAFII cells (Figure 4.6 - A1). Ocln and c-Yes co-localised in large aggregates at the cell membrane and in regions adjacent to the nucleus but these occurred to a lesser extent than c-Yes and YAP and showed similarities to the Ocln and YAP distribution: mostly cytoplasmic interactions (Figure 4.6 – A2). Ocln and YAP co-localised essentially at the external facing surfaces of these cell aggregates, although less striking relative to Ocln/c-Yes and c-Yes/YAP interactions. Interestingly, higher magnification analysis showed TEAD/Ocln co-localisations to be diffusely distributed throughout the cytoplasm and many of these interactions were incomplete in their overlap (Figure 4.6 - A4), unlike the more complete overlay observed for c-Yes/Ocln, c-Yes/YAP, and YAP/Ocln co-localisations (Figure 4.6 - A1-A3). There was also a similar cytoplasmic and possibly some nuclear co-localisation observed for Ocln and TEAD, with limited interactions at the cell surface (Figure 4.6 - A4). Following this, the interactions between Ocln and Hpo pathway elements

were examined in the AsPc-1 cell line, known for its aggressive growth and inability to form functional TJ (Tan and Chu, 1985). When grown at low cell density as previously described for HPAFII cells (Figure 4.6), AsPc-1 cells expressed Ocln at levels comparable to or even greater than those observed in HPAFII cells with some immunofluorescence being associated with the plasma membrane (Figure 4.7-A1). Previously shown to be associated with carcinogenesis (Barraclough et al., 2007), the interactions between YAP and the proto-oncogene c-Yes appeared to occur mostly in the cytoplasm and not in the nucleus. Ocln and c-Yes co-localised predominantly at the membrane similarly to that observed in HPAFII cells (Figure 4.7 - A2). YAP and Ocln interactions occurred almost exclusively in large aggregates at the plasma membrane and in the nucleus of AsPc-1 cells, different from that observed for HPAFII cells (Figure 4.7 - A3). This was further confirmed by an immunoprecipitation assay that showed an interaction between YAP and Ocln at the cell membrane of aggressively growing AsPc-1 cells but not in HPAFII cells (Figure 4.8). Furthermore, another striking difference between HPAFII and AsPc-1 cells was primarily related to YAP and TEAD distribution with a much greater relative expression in the nucleus of the aggressively growing AsPc-1 cells (Figure 4.7 - A4). TEAD/Ocln interaction was almost non-existent and failed to show cytoplasmic co-localisations as observed in HPAFII cells.


A
A1
YAP
DAPI
c-Yes
Merged
A2
Ocln
DAPI
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YAP
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A4
Ocln
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TEAD
Merged

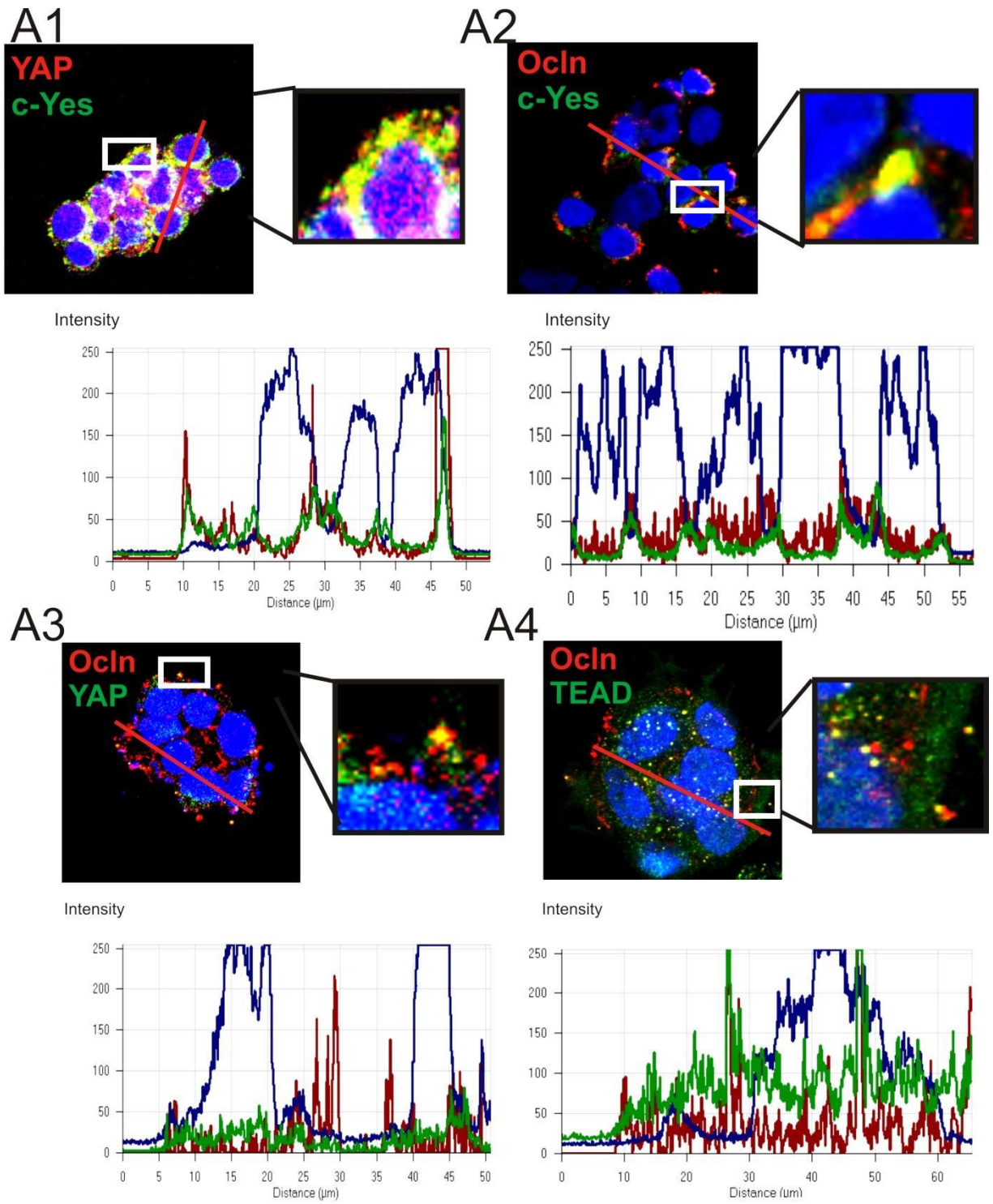
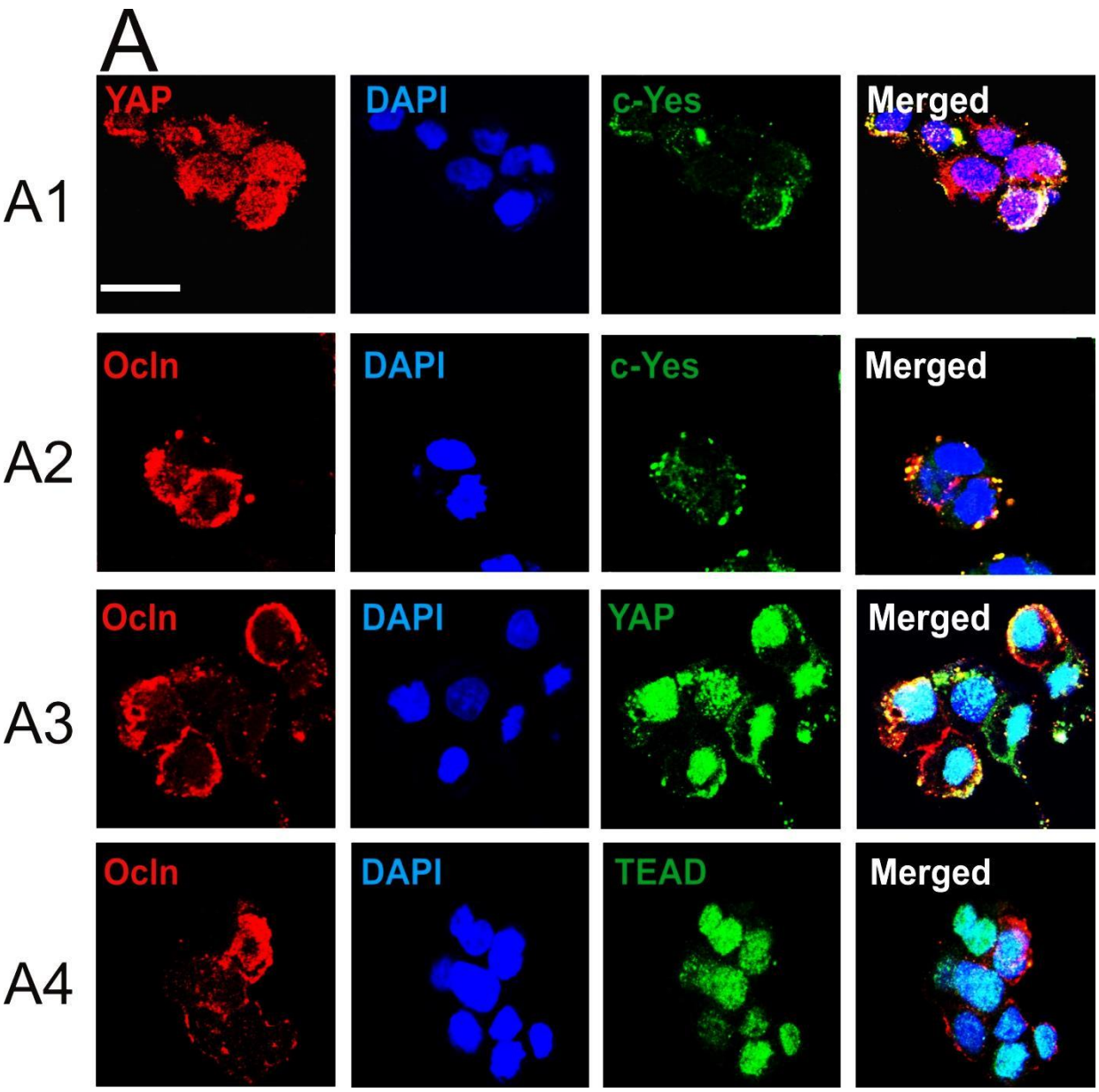


Figure 4.6 Distribution and Co-localisation of YAP, c-Yes, Ocln, and TEAD in HPAFII cells *in vitro*. (A) Immunofluorescent distribution and confocal co-localisation of YAP and c-Yes **(A1)**, Ocln and c-Yes **(A2)**, Ocln and YAP **(A3)**, and Ocln and TEAD **(A4)**. Higher magnifications of co-localisations are presented **(A1-4)**. Graphs showing overlapping fluorescence intensity are presented below with the associated micrograph with red line denoting path of analysis; scale bar, 20 µm.


A
A1
YAP
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c-Yes
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Ocln
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YAP
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TEAD
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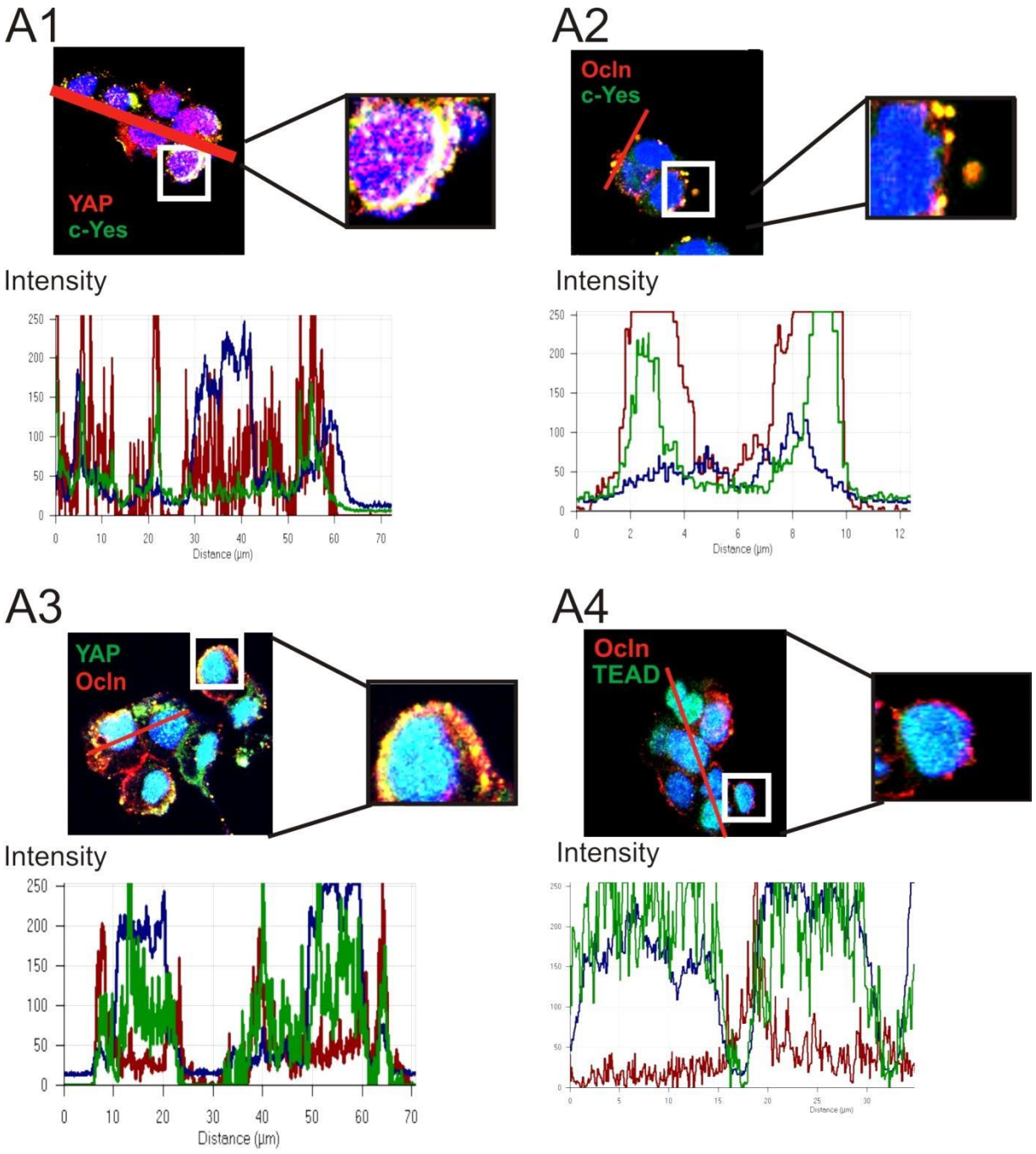

Figure 4.7 Distribution and Co-localisation of YAP, c-Yes, Ocln, and TEAD in AsPc-1 cells *in vitro*. (A) Immunofluorescent distribution and confocal co-localisation of YAP and c-Yes **(A1)**, Ocln and c-Yes **(A2)**, Ocln and YAP **(A3)**, and Ocln and TEAD **(A4)**. Higher magnifications of co-localisations are presented (A1-4). Graphs showing overlapping fluorescence intensity are presented below with the associated micrograph with red line denoting path of analysis; scale bar, 20 µm.

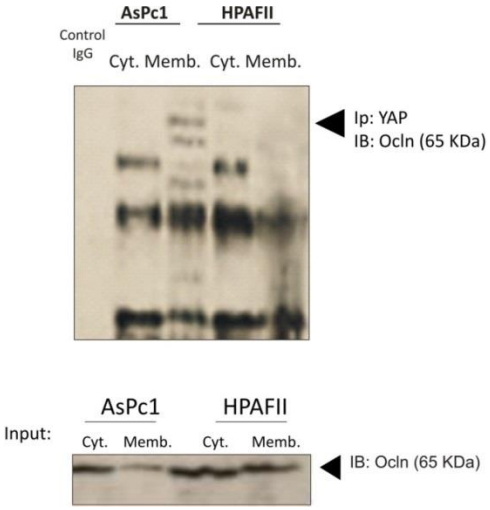

Figure 4.8 Co-localisation of Ocln and YAP at the cell membrane of aggressive growing AsPc-1 cells. Immunoprecipitation of YAP followed by an immunoblot analysis for Ocln in cytoplasmic and membrane fractions of AsPc-1 and HPAFII cells. ***Abbreviations: Cyt (Cytosolic fraction); Memb (Membrane fraction).***

4.3.4 Dobutamine treatment induced the redistribution of functional Ocln in HPAFII cells

Subcellular localisation of YAP is critical for Hpo pathway regulation (Boggiano and Fehon, 2012; Zhao et al., 2007). The Hpo pathway responds to cell density and regulates YAP phosphorylation, preventing its translocation into the nucleus where it functions as a transcriptional co-activator (Zhao et al., 2007). As previously mentioned, Bao and co-workers suggested that dobutamine was able to induce YAP phosphorylation at Ser^{127} inhibiting YAP-mediated gene transcription in osteoblastoma cells (Bao et al., 2011). Since the Hpo pathway is activated by different upstream molecular signals including cell-cell contact and cell polarity molecules (Yu et al., 2012), we asked whether dobutamine treatment could affect the cellular distribution or function of the TJ marker Ocln based upon the observed YAP/Ocln interactions and as a consequence of shifting YAP cell distribution. To test this, HPAFII cells were subjected to 10 and 20 μM dobutamine as a single treatment

during 24 h. HPAFII cells grown at high cell density on plastic to provide extensive cell-cell contacts and treated with dobutamine resulted in a dose-dependent shift of YAP immunofluorescence from the nucleus to the cytoplasm relative to controls. This event was associated with Ocln stabilization at the plasma membrane (Figure 4.9 – A). Western Blot analysis of isolated cytoplasm and nuclear fractions (Figure 4.9 - B) demonstrated a striking reduction in nuclear YAP content following dobutamine treatment, presumably through its redistribution to the cytoplasm where it is phosphorylated and degraded (Chan et al., 2011). Dobutamine treatment in this cell system also produced a dramatic loss of nuclear TEAD with the remaining TEAD being notable for its apparent co-localisations with Ocln at the plasma membrane in a punctate pattern (Figure 4.10 – A, white arrows pointing at co-localisation regions and Figure 4.10-B).

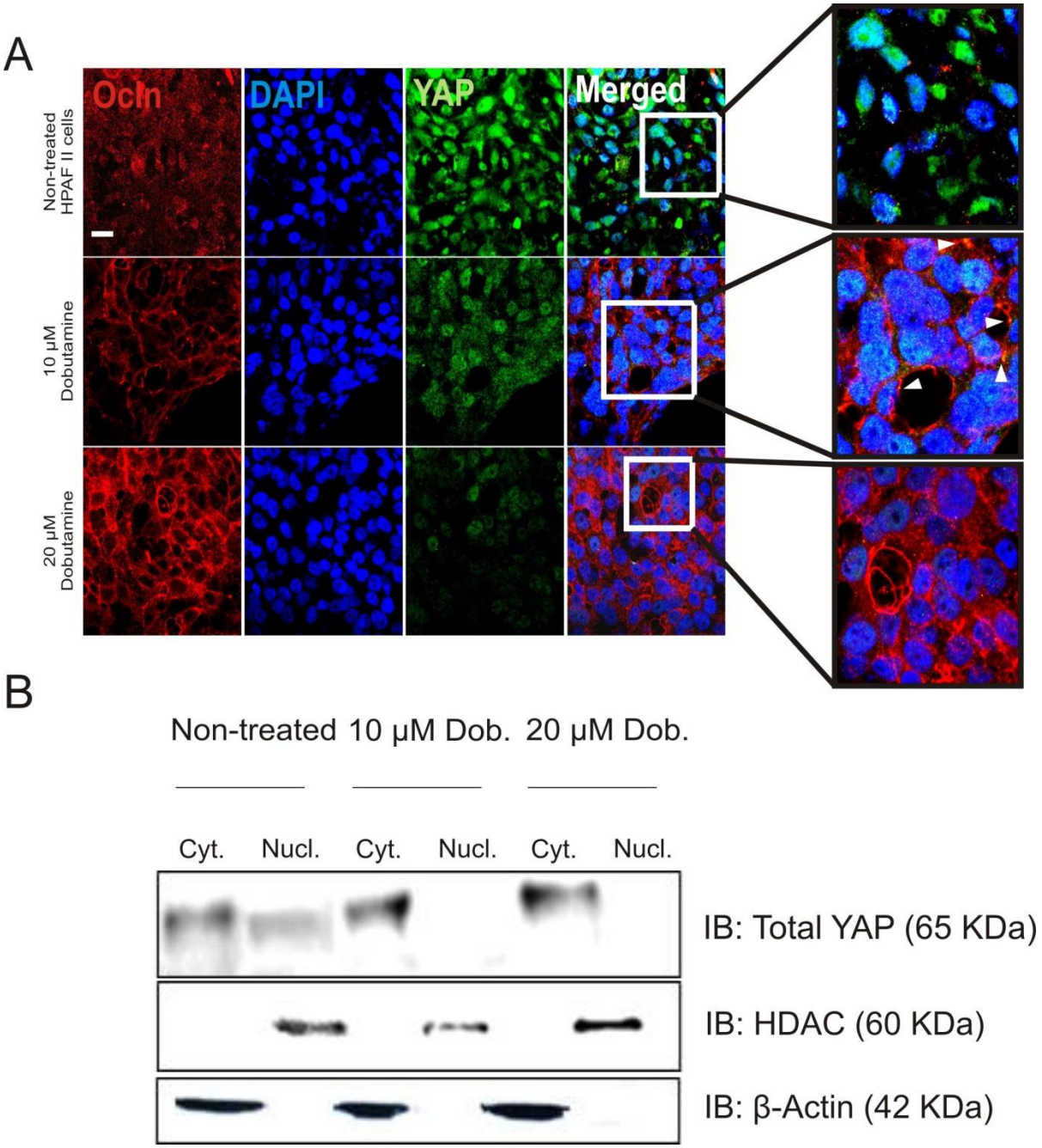

Figure 4.9 Dobutamine induces YAP translocation from the nucleus and stabilization of Ocln at the cell membrane of HPAFII cells. (A) Confocal immunofluorescence microscopy showing YAP and Ocln distribution in HPAFII cells. Cells treated with 10 or 20 µM dobutamine for 24 h were similarly analysed for YAP and Ocln cell distribution. Scale bar, 20 µm; **(B)** Immunoblot analysis of total YAP in nuclear and cytoplasmic fractions of HPAF II cells as a consequence of 10 or 20 µM dobutamine (Dob) exposure. HDAC (histone deacetylase) and β-actin were used as loading controls for nuclear and cytoplasmic fractions, respectively. ***Abbreviations: Dob. - Dobutamine; Cyt - Cytoplasm; Nucl. - Nucleus.***

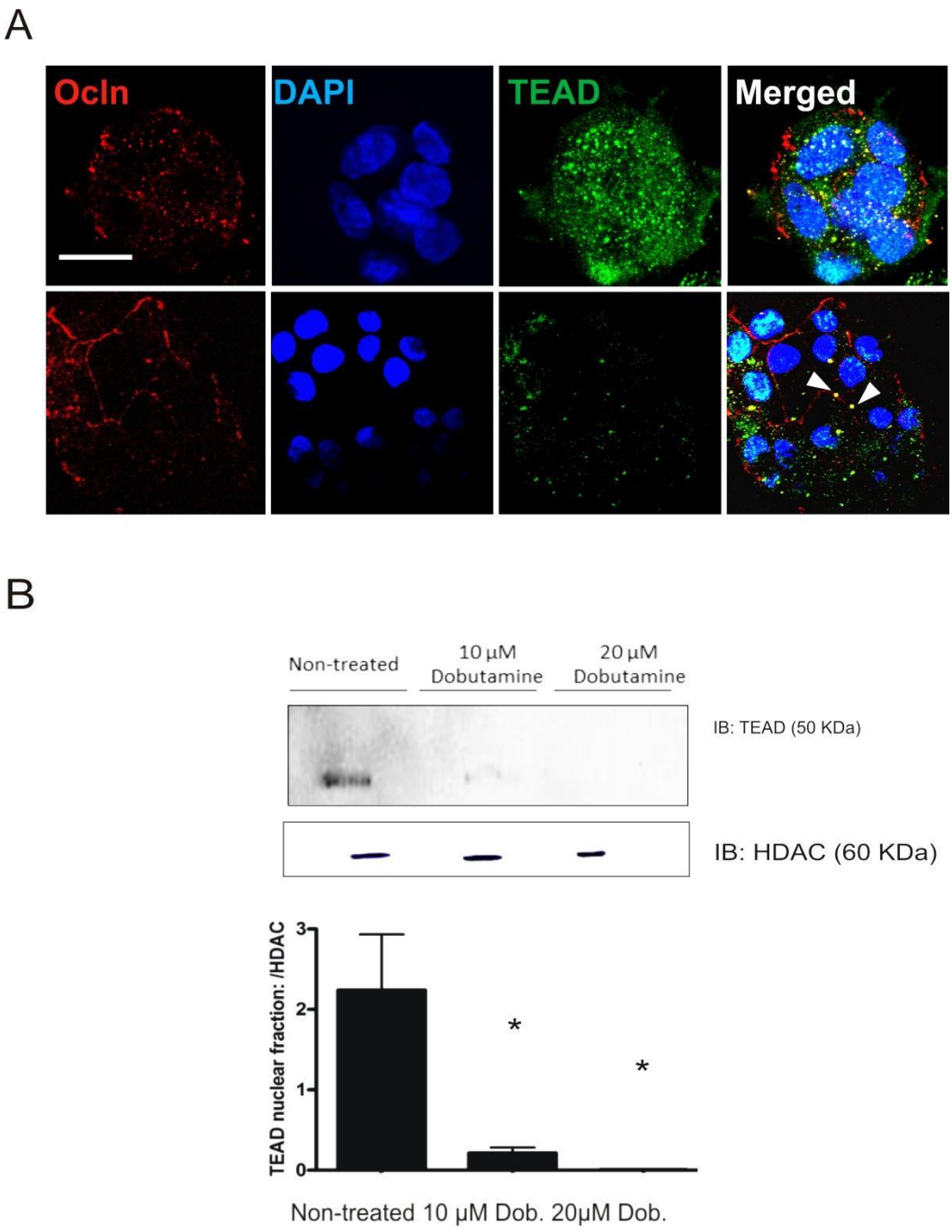

Figure 4.10 Dobutamine induces TEAD translocation from the nucleus and stabilisation of Ocln at the cell membrane of HPAFII cells. (A) Confocal immunofluorescence showing TEAD and Ocln distribution in HPAFII cells with control buffer addition versus 20 µM dobutamine for 24 h; scale bar, 20 µm. **(B)** Immunoblot analysis of TEAD in the nuclear fractions of HPAFII cells as a consequence of 10 or 20 µM dobutamine exposure. Quantification of TEAD expression values are mean of 3 independent experiments ± SEM; *p < 0.05. ***Abbreviations: Dob. – Dobutamine.***

4.3.5 Dobutamine-induced redistribution of Ocln has functional consequences

Past studies showed that Ocln immunoprecipitates and co-localises with non-receptor tyrosine kinase c-Yes (Chen and Lu, 2003). Further to this, the same study showed that treatment of MDCK cells with a c-Yes inhibitor disrupted the Ocln/c-Yes complex inhibiting the phosphorylation of Ocln required for TJ formation (Chen et al., 2002b). Initially, we sought to analyze c-Yes distribution in pancreatic tissue biopsies from patients diagnosed with CP and PDAC compared to normal pancreatic tissue sections. In normal (non-cancerous, non-inflamed) pancreas, c-Yes was distributed in the cytoplasm and in small amounts at the apical plasma membrane of ducts as pointed by the black arrows (Figure 4.11). A moderate to strong nuclear c-Yes staining was observed in pancreatic cancer tissue biopsies and a strong signal was also detected at the apical surface of epithelial cells as indicated by the black arrows (Figure 4.11), which agrees with the distribution of this non-receptor tyrosine kinase in AsPc-1 cells (Figure 4.7 - A1, A2).

Next, it was investigated whether dobutamine could affect c-Yes cellular distribution to align it with Ocln expression at these external faces; c-Yes distribution was monitored after exposing cells to 10 and 20 µM dobutamine during 24 h. Treatment of HPAFII cells grown at low cell density caused a dose-dependent shift of c-Yes from the cytoplasm to the plasma membrane. Concomitant with this shift, there was an increase in co-localisation and association between c-Yes and Ocln at the plasma membrane (Figure 4.12 - A, B). Presumably this phenomenon happened through translocation of YAP from the nucleus to the cytosol. These results are consistent with previous findings suggesting that c-Yes is associated with Ocln at assembling TJ structures but is dissociated from Ocln at times when TJ structures are disassembling (Chen et al., 2002b). Thus, suppression of YAP

translocation to the nucleus appears to allow for more c-Yes-Ocln associations in HPAFII cells that are able to polarise.

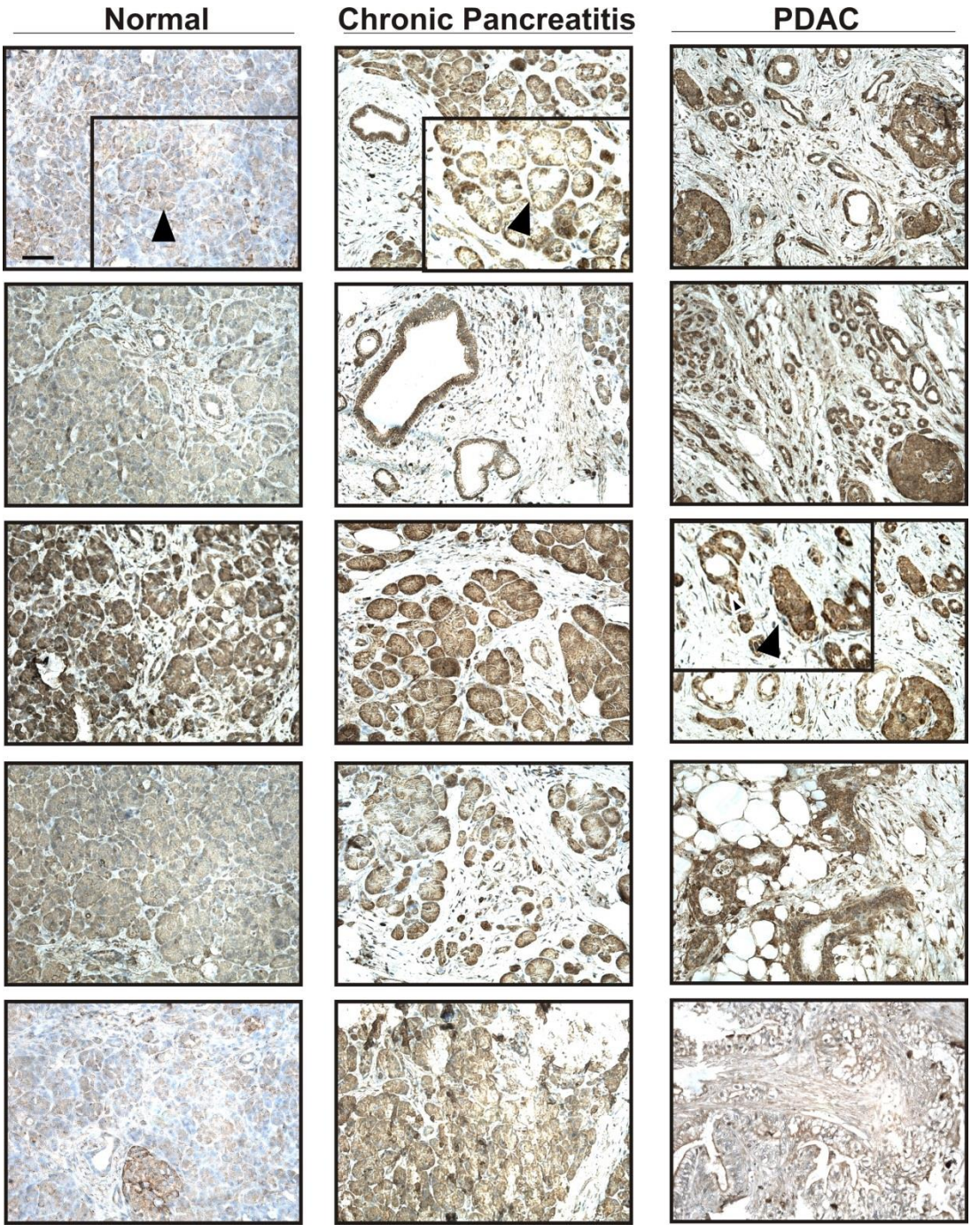

Figure 4.11 Nuclear c-Yes distribution increases progressively in CP and in PDAC human; Immunohistochemistry analysis of 15 human pancreatic biopsies (5 patients with normal pancreas, 5 patients with CP and 5 patients with PDAC) immunostained for c-Yes; Insets with higher magnification of specific tissue regions are presented and black arrows are pointing at specific immunolabelling; scale bar, 50 µm.

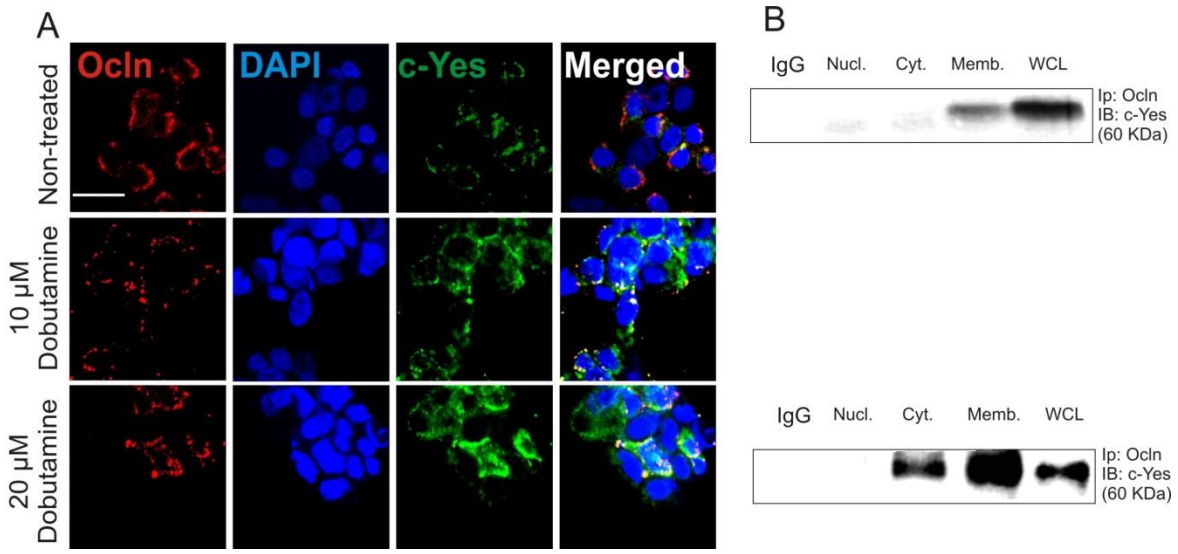

Figure 4.12 Redistribution of Ocln and c-Yes in HPAFII cells following dobutamine treatment. **(A)** Confocal microscopy showing c-Yes and Ocln distribution in HPAFII cells. Cells were treated with 10 or 20 µM dobutamine for 24 h period prior to c-Yes and Ocln cell distribution analysis. Scale bar, 20 µm. **(B)** Immunoprecipitation of Ocln followed by an immunoblot analysis for c-Yes in nuclear, cytoplasmic, nuclear and membrane fractions of non -treated HPAFII cells (top panel) and after 20 µM dobutamine exposure (bottom panel). ***Abbreviations: Nucl (Nuclear fraction); Cyt (Cytosolic fraction); Memb (Membrane fraction); WCL (Whole cell lysate).***

Besides Ocln, two other MARVEL family proteins localize to TJ structures: MARVELD2 (Tric) and MARVELD3 (Md3) (Mariano et al., 2011). As previously mentioned, Raf1-mediated induction EMT was associated with the loss of functional TJ structures and EMT could be reversed by the re-introduction of Ocln (Li and Mrsny, 2000). Similar to Ocln, down-regulation of Md3 is associated with EMT in pancreatic cancer cells; Ocln and Md3 strongly co-localise at bi-cellular contacts (Kojima et al., 2011). Knock-down studies of Tric, localised to tri-cellular contacts, suggest it to have a more directed role at the size-selective property of TJ barriers rather than EMT events (Mariano et al., 2011).

As Tric is involved in the establishment of functional TJs, the impact of dobutamine on Tric organization in HPAFII cells that had begun forming cell-cell contacts was examined. Untreated HPAFII cells demonstrated co-localised Ocln-Tric that was predominantly

distributed at both internal cell-cell contact sites and at cell surfaces of the external face of these cell colonies (Figure 4.13 – A). Dobutamine treatment resulted in the exclusion of Tric from bi-cellular contact faces that were not dominated by Ocln. Interestingly, Ocln-Tric was still observed to co-localise at the externally directed cell surfaces of these cell colonies. Immunoprecipitation studies confirmed that following dobutamine treatment, Tric associations with Ocln at the cell membrane were diminished compared to non-treated cells (Figure 4.13 –B). Our data shows that Tric expression shifts from bi-cellular to tri-cellular contacts after treatment with dobutamine results in the enhanced expression of Ocln. The lack of Tric restriction at externally directed cell surfaces suggests that cell-cell contacts are required in these HPAFII cells to allow Ocln to affect Tric localisation at the plasma membrane. Our data is consistent with that of others who have described the ability for Ocln to drive Tric from bi-cellular to tri-cellular contact sites (Ikenouchi et al., 2005). What is novel about this finding is that suppression of Hpo pathway signaling allowed for this segregation of Tric localisation. Here, it is important to note that c-Yes was also observed to localize more predominantly with Ocln at bi-cellular contacts than with this kinase at externally directed cell surfaces of these cell colonies.

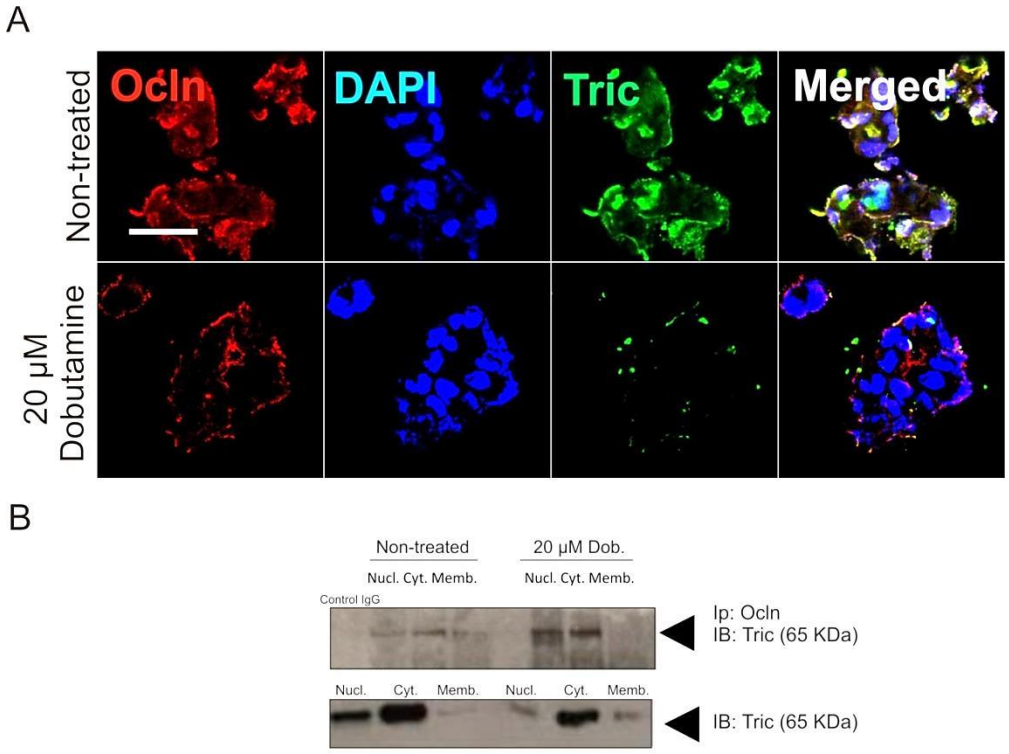

Figure 4.13 Restriction of Tric to tri-cellular contacts after dobutamine treatment. (A) Confocal images showing distribution of Ocln and Tric in HPAFII cells treated with 20 μM dobutamine for 24 h. Scale bar, 20 μm. **(B)** Immunoprecipitation of Ocln followed by an immunoblot analysis for Tric in nuclear, cytoplasmic and membrane fractions of HPAFII cells following 20 μM dobutamine exposure. ***Abbreviations: Nucl (Nuclear fraction); Cyt (Cytosolic fraction); Memb (Membrane fraction).***

4.3.6 Dobutamine accelerates and enhances epithelial cell apical-basal polarization

We next asked if the translocation of Ocln from the cytoplasm to the plasma membrane as a result of dobutamine treatment could be associated with functional TJ structures (Figure 4.9). Confluent cultures of HPAFII cells grown on plastic and treated with dobutamine demonstrated subtle changes in phenotype consistent with an increasing extent of cell-cell contacts without striking differences in cell number, size, or shape (Figure 4.14 - A-C). HPAFII cells were then grown as confluent monolayers on permeable supports to allow their complete apical-basal polarization; this resulted in the establishment

of polarised sheets with TER values of ~300-500 Ω.cm^2 by day 8 of culture (Figure 4.14-D). Addition of dobutamine accelerated and enhanced the TER values of HPAFII cell sheets in a dose-dependent manner with TER values increasing to as high as ~4,000 Ω.cm^2 (Figure 4.14 - D) but plateauing at ~1,700 Ω.cm^2 (Figure 4.14 - E). Ocln protein distribution in polarised sheets of HPAFII cells, assessed by confocal immunofluorescence, demonstrated the localisation of this protein across the apical surface and at TJs located at the apical neck of adjacent cells; dobutamine treatment resulted in an apparent increase in total Olcn levels and a consolidation of this protein at TJs relative to its expression across the apical surface (Figure 4.14 - F). Immunoblot analysis verified that dobutamine treatment increased total cellular Ocln levels (Figure 4.14 – G and H).

Further to this, the condition of Ocln redistribution as a consequence of dobutamine treatment was also investigated. Immunoblot analysis showed that Ocln associated with the membrane fraction of polarised HPAFII cells treated with dobutamine had an increased relative molecular weight (Figure 4.14 - I), consistent with an increased phosphorylation status (Dorfel and Huber, 2012).

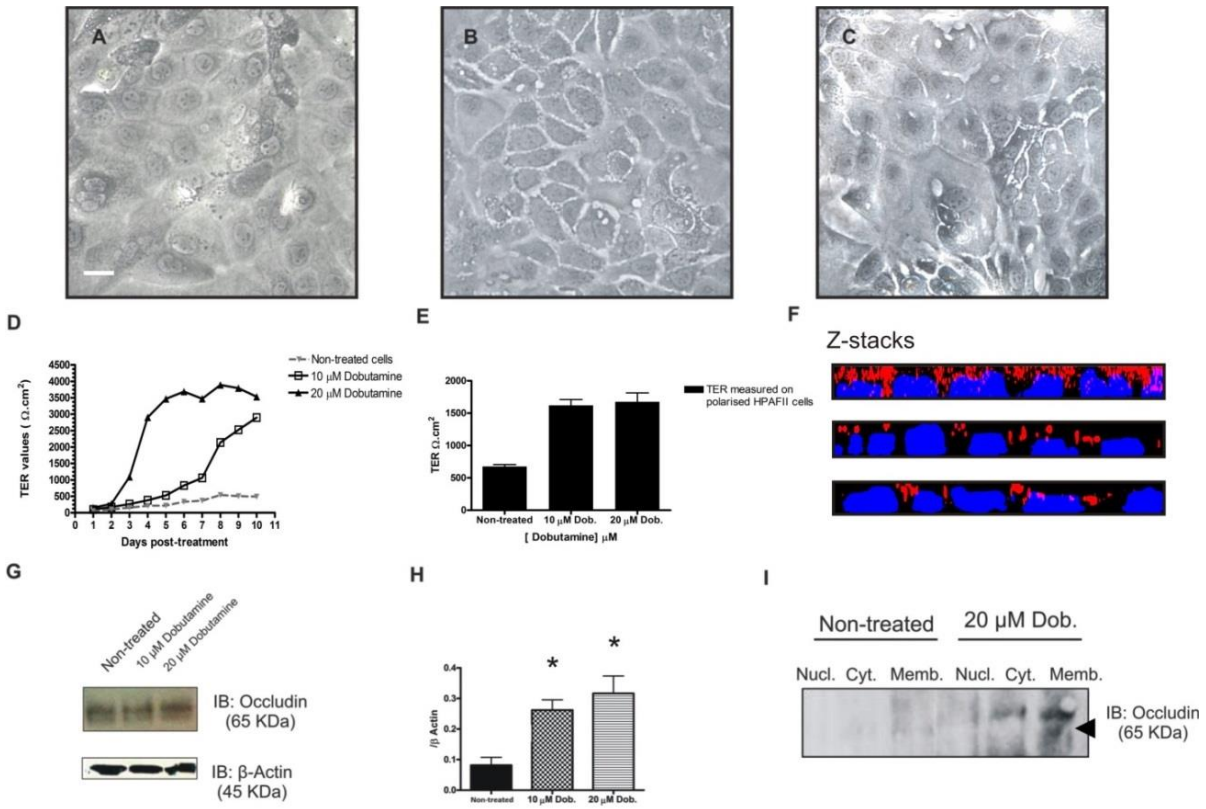

Figure 4.14 Dobutamine treatment of polarised cell sheets of HPAFII cells. Bright field light micrograph of confluent HPAFII cells grown on plastic without treatment **(A)**, or after 24 h exposure to 10 μM dobutamine **(B)** or 20 μM dobutamine **(C)**; scale bar, 50 μm. TER values of HPAFII cell sheets cultured on permeable supports in the absence or presence of dobutamine **(D)**; TER values acquired on day 10 – note this is a separate study from data shown in **(E)**. Z-stacks of non-treated polarised HPAF II cells (top panel) or HPAFII monolayers exposed to 10 μM or 20 μM of dobutamine (middle and bottom panel respectively) red (Ocln), blue (cell nuclei) **(F)**; Immunoblot for total Ocln in HPAFII cell monolayers at day 10 **(G)**; Quantification of total Ocln in HPAFII cells at day 10 culture comparing non-treated with cells exposed to dobutamine; values are mean of 3 independent experiments ± SEM; * $p<0.05$ **(H)** Immunoblot for Ocln in HPAFII cells present in isolated fractions before and after treatment with dobutamine **(I)**. ***Abbreviations: Nucl (Nuclear fraction); Cyt (Cytosolic fraction); Memb (Membrane fraction).***

Subsequently, the enhanced level of Ocln at the plasma membrane was also observed to be associated with the formation of rosettes that resemble primitive lumen organizations in HPAFII cells cultured in a 2-D cell model (Figure 4.15).

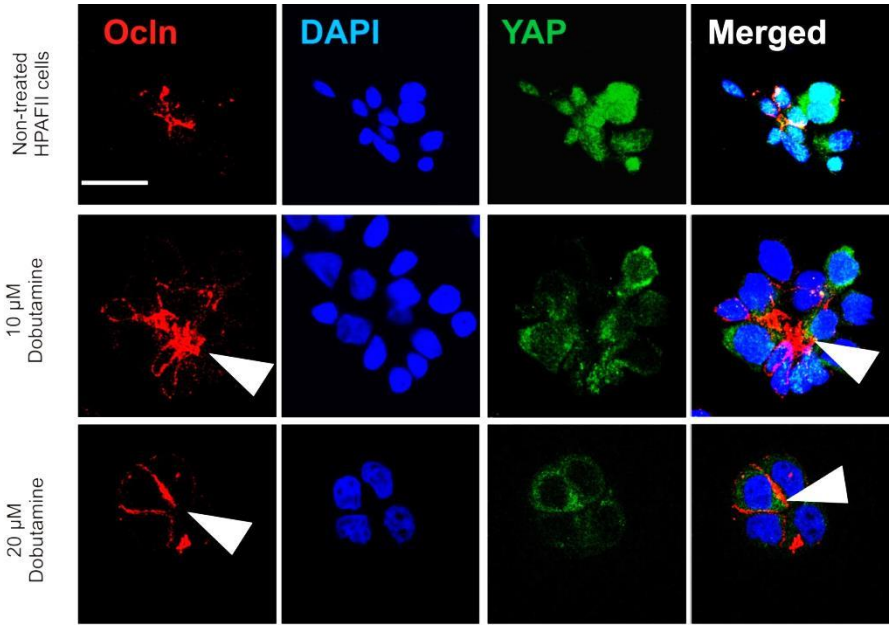

Figure 4.15 Formation of "luminal-like" structures after HPAFII cells were treated with dobutamine for a 24 h period; Confocal images showing a progressive decrease in YAP nuclear signal and the formation of "rosette" structures (pointed by the white arrows) after HPAFII cells were treated with dobutamine; scale bar, 20 µm.

4.3.7 Dobutamine affects cellular distribution of YAP, Ocln, c-Yes and TEAD in AsPc-1 cells

Dobutamine actions were then examined in an epithelial pancreatic cancer cell line that does not demonstrate apical-basal polarization *in vitro*: AsPc-1. Treatment of AsPc-1 cells with dobutamine increased Ocln expression and directed its distribution to the plasma membrane, similar to that observed in HPAFII cells (Figure 4.16). An increase in the extent of c-Yes-Ocln interactions in large structures could also be observed at or near the plasma membrane, similar to HPAFII cells (Figure 4.17). Western Blot also confirmed increased Ocln and c-Yes interactions at the cell membrane after dobutamine treatment. As expected, dobutamine caused a loss of nuclear YAP and TEAD in AsPc-1 cells as seen with HPAFII cells (Figure 4.18), but in these cases there were no observed YAP-Ocln or TEAD-Ocln co-localisations at the cell surface as detected in HPAFII cells (Figure 4.10-A).

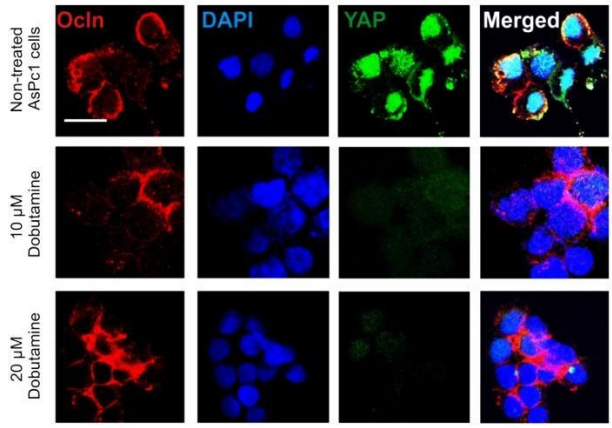

Figure 4.16 YAP trans-locates to the cytoplasm and Ocln formation gets stabilized at the cell membrane of AsPc-1 cells; Confocal images showing YAP trans-location from the nucleus to the cytoplasm and Ocln formation at the cell membrane after cells were treated with 10 and 20 µM dobutamine for a 24 h period compared to non-treated cells. Scale bar, 20 µm.

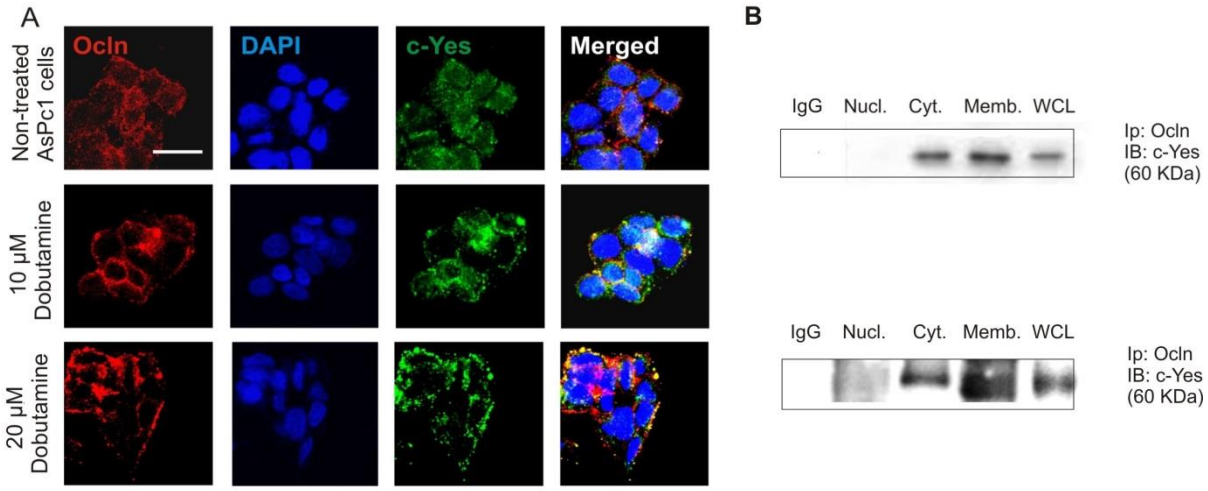

Figure 4.17 c-Yes trans-locates from the cytosol to the cell membrane of AsPc-1 cells. **(A)** Confocal images showing co-localisation of Ocln and c-Yes as cells are treated with 10 and 20 µM dobutamine for a 24 h period. Scale bar, 20 µm; **(B)** Immunoprecipitation of Ocln followed by an immunoblot analysis for c-Yes in nuclear, cytoplasmic, and membrane fractions of non-treated AsPc-1 cells (top panel) and 20 µM dobutamine treated (bottom panel). ***Abbreviations: Nucl. – Nuclear fraction; Cyt. – Cytosolic fraction; Memb. – Membrane fraction; WCL – Whole cell lysate input.***

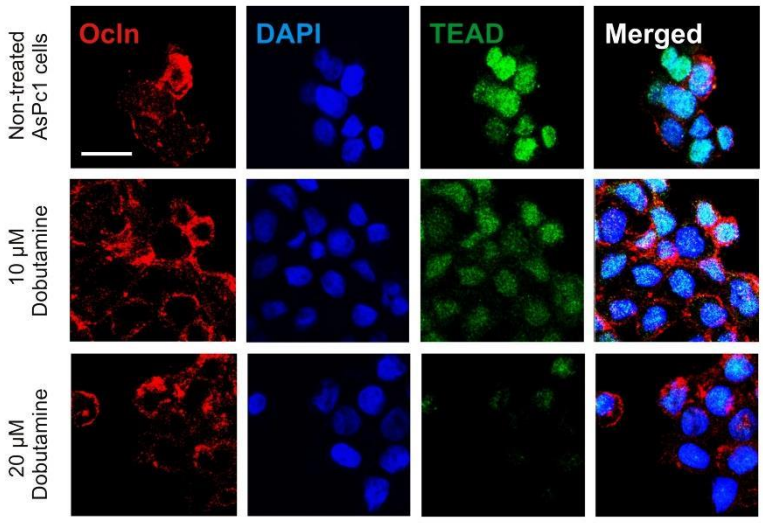

Figure 4.18 Nuclear TEAD signal gets reduced after dobutamine treatment in AsPc-1 cells; Confocal images showing a progressively weaker nuclear TEAD labelling as cells are treated with 10 and 20 µM dobutamine for a 24 h period. Scale bar, 20 µm.

4.3.8 Dobutamine treatment affected differently CRB3 cell distribution and the extent to which it co-localised with Ocln in HPAFII and AsPc-1 cell lines

The Crumbs Polarity Complex (CPC) is involved in the monitoring of apical to basolateral polarity of an epithelial cell (Tepass, 1996), however this complex is not known to establish robust cell-cell contacts that could provide the basis for monitoring lateral cell density that precedes the formation of TJ structures. CRB3 functions as a membrane element that participates in the polarity complex through interactions with PAR-6 via TJ-associated PALS1 and is associated with TJs at the apical surface of epithelial cells (Lemmers et al., 2004). Thus, CRB3 would be a likely candidate to function as an external sensor for cell-cell contact events associated with apical-basal polarity events. We asked the question of whether the potential interaction of Ocln with polarity complexes might be integrated into the way this MARVEL protein interacts with YAP. Immunofluorescence studies demonstrated CRB3 and Ocln to be separately dispersed through the cytoplasm and

at the plasma membrane of non-polarised HPAFII cells. Some co-localisation between CRB3 and Ocln labelling was observed in the nucleus of these cells. Most strikingly, CRB3 distribution and interactions with Ocln differed between HPAFII and AsPc-1 cells. In HPAFII, CRB3-Ocln co-localisations were limited to some overlapping fractions of small vesicles and were not affected substantially by dobutamine treatment (Figure 4.19-A). Oppositely, CRB3-Ocln interactions were extensive in AsPc-1 cells, which shifted from large aggregates near the plasma membrane to a more uniform distribution across the plasma membrane following dobutamine treatment (Figure 4.19-B). These results suggested that CRB3 redistribution followed that of Ocln changes induced by dobutamine in AsPc-1 cells but not in HPAFII cells.

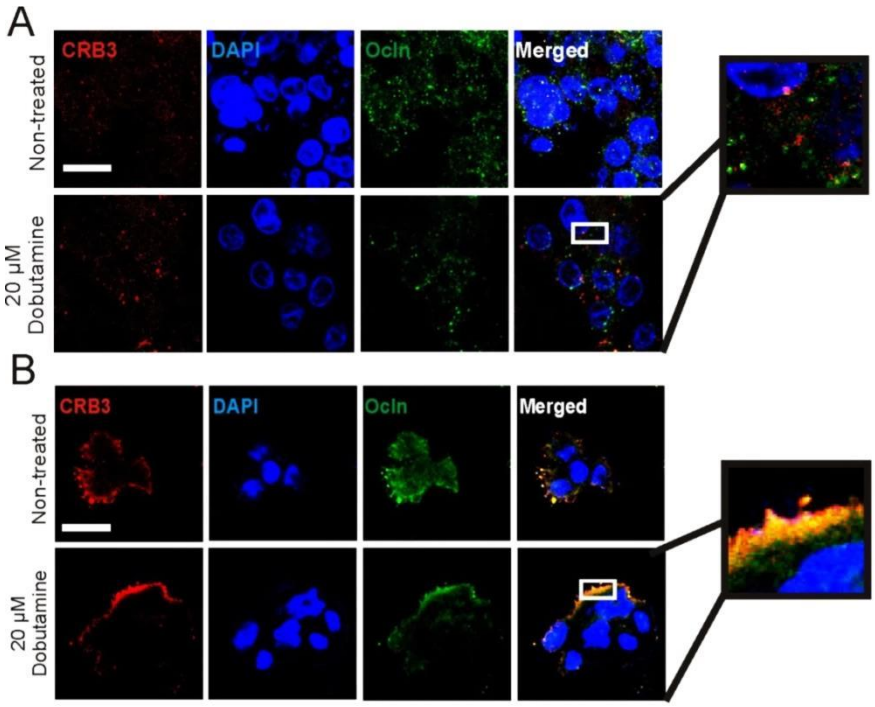

Figure 4.19 CRB 3 does not co-localise with Ocln at the cell membrane of HPAFII cells. Confocal images showing different cell distribution of CRB3 and Ocln in HPAFII **(A)** and in AsPc-1 **(B)**. Scale bar, 20 μm.

4.4 Summary of results

- Ocln tissue staining decreases and YAP tissue staining increases with pancreatic cancer progression;
- Nuclear TEAD tissue expression increases in PDAC;
- Ocln co-localises with Hpo pathway elements in HPAFII and AsPc-1 cells grown at low density;
- Dobutamine treatment induced the redistribution of functional Ocln in HPAFII cells;
- Dobutamine affects cellular distribution of YAP, Ocln, c-Yes and TEAD in AsPc-1 cells;
- Dobutamine treatment differently affected CRB3 cell distribution and the extent to which it co-localised with Ocln in HPAFII and AsPc-1 cell lines.

4.5 Discussion

The Hpo pathway provides important growth regulation of polarizing epithelia through an interaction between the Crumbs complex and YAP (Varelas et al., 2010). These studies were a landmark in that they set YAP at the location of apical neck of polarizing epithelial cells where interactions with proteins such as PALS1, PATJ, MUPP1, LIN7C, and AMOT could couple cell density regulation with the establishment of TJs. The Crumbs complex, however, does not affect cell-cell junction formation (Varelas et al., 2008). Despite the fact of interacting at the cytoplasmic surface of TJ structures (Tepass, 1996), Crumbs complex elements do not contain a component that directly establishes extracellular protein-protein interactions that might sense polarity status with an adjacent epithelial cell .

These observations have naturally raised the question about the physiological function of Ocln, suggesting it not to play an essential role in epithelial barrier function *in vivo*. Recent studies, however, support the potential for another TJ-associated MARVEL protein member normally restricted to tri-cellular interaction sites, Tric, to replace absent Ocln at bi-cellular contacts (Takasawa et al., 2013). This replacement does not provide an equivalent correction, however, might provide an explanation for the Ocln knockout mice. While $Ocln^{-/-}$ mice demonstrate morphologically and functionally normal TJs, reach adulthood and are born with no abnormal phenotype in the expected Mendelian rations, they exhibit a complex phenotype that includes reduced growth rates, brain calcifications, testicular atrophy, loss of cytoplasmic granules in salivary gland, striated duct epithelial cells, and thinning of compact bone (Saitou et al., 2000).

Despite the controversy, there is a relationship between CP and PDAC (Logsdon et al., 2010). Thus, our comparisons between pancreatic epithelial cell lines capable of polarizing *in vitro* (HPAFII to emulate a non-cancerous format) or being incapable of this process (AsPc-1 to emulate a cancerous format) were used to examine potential differences that might be relevant to pathological states.

The data presented in this chapter initially described Ocln and YAP distribution in CP and PDAC biopsy samples obtained from a limited set of patients. In normal tissue, Ocln localised primarily at the cytoplasm and at the apical surface of ducts. In PDAC tissue, Ocln staining appeared to be substantial but did not distribute at the plasma membrane, consistent with past studies reporting a correlation between the loss of Ocln and pancreatic cancer progression (Tan et al., 2004) (Figure 4.1-top panel, Figure 4.2). Loss of Ocln expression or disruption of its normal cellular organization was found to be associated with epithelial cell transformation (Figure. 4.1-top panel), consistent with several studies reporting

the down-regulation of Ocln and its correlation with cancer progression (Martin et al., 2010; Osanai et al., 2007; Tobioka et al., 2004a).

In this limited set of biopsies, YAP was mostly detected in the cytoplasm of normal acinar and ductal cells with minimal nuclear staining observed. In CP samples and more markedly in cancer, a strong nuclear signal could be observed. In cancer biopsies, strong YAP staining was detected in PanIN lesions (Figure 4.1 – bottom panel, Figure 4.3); nuclear expression of YAP was found to be associated with cytoplasmic expression (Figure 4.3). This might indicate a possible mechanism involved in the alteration and enhancement of YAP activity observed in some epithelial cancers. Malignant cells might produce an excess of YAP during genomic amplification overwhelming the stable environment essential for normal cell function. Hence, this leads to an abnormal cytoplasmic accumulation of YAP establishing a dynamic equilibrium between the two pools of proteins for nuclear translocation. Also, another possibility is the fact that in cancer, the stability of YAP protein might be altered as a consequence of an anabolic state that generates an imbalance between protein synthesis and protein degradation. Overall, YAP was predominantly distributed in the nucleus however some cytoplasmic expression could be observed in both inflamed and cancerous conditions. These findings are supported by past studies reporting YAP to be amplified in a variety of human cancers such as oral cancer (Baldwin et al., 2005) and oral squamous cell carcinoma (Snijders et al., 2005), hedgehog-associated medulloblastomas (Fernandez-L et al., 2009), colon, lung and ovarian serous cystadenocarcinoma (Steinhardt et al., 2008), hepatocellular carcinomas and breast cancers (Overholtzer et al., 2006; Zender et al., 2006).

High levels of nuclear YAP has been associated with a worse overall survival and shorter disease-free progression particularly in human esophageal squamous cell carcinoma

(ESCC) and in adenocarcinoma of the cervix (Imoto et al., 2001; Tang, 2002). This is consistent with the known cellular function of YAP protein: YAP is localised in cytoplasm when it is inactivated and it gets trans-located into the nucleus when gets activated (Zhao et al., 2007). Even though the immunohistochemistry staining was not quantified by an experienced pathologist, it was quite clear overall YAP labelling appeared to be stronger in CP relative to normal tissue particularly the nuclear staining. Cancerous tissue had a considerably greater amount of YAP labelling compared to normal or inflamed pancreas biopsies (Figure 4.3).

Given the role of PSCs in modulating pancreatic tumour microenvironment (Omary et al., 2007), YAP expression levels were analysed in PSCs lysates collected from three PDAC patients and two CP patients (Figure 4.4). Absent YAP levels were observed in CP samples whereas in PDAC samples, at least two patients showed a positive signal. These results did not fully match early observations reporting a weak to moderate YAP staining intensity in inflamed tissue. However, due to the limited sample size and also the technical difficulties in obtaining PSC aspirates from patients, no further conclusions can be taken on this experiment.

Studies have addressed the role of YAP overexpression in stimulating proliferation (Camargo et al., 2007; Dong et al., 2007; Zhao et al., 2007). Several transcription factors, including Runx2, ErbB4, p73 and TEAD in particular, have been reported to interact with YAP (Basu et al., 2003; Vassilev et al., 2001). TEAD distribution levels were analysed in normal, inflamed and cancerous pancreatic biopsies. PDAC tissue showed some cytoplasmic staining but more strikingly an extensive nuclear localisation in comparison to normal or inflamed pancreas (Figure 4.5). Zhao and co-workers suggested TEAD to be one of the most potent YAP targets from a transcription-activity based screen. In addition, CTGF

was identified as a direct target gene resulting from the interaction between nuclear YAP and TEAD. Moreover, knock-down of CTGF was found to abrogate YAP-stimulated cell growth reducing significantly YAP-induced colony formation (Zhao et al., 2008).

Sensing the extent of mature cell-cell contacts at the time of polarity induction is critical for the establishment and maintenance of epithelial sheet composition and characteristics. The interactions between Hpo signaling pathway and Ocln would provide a mechanism to measure the extent of lateral membrane interactions and the organization of cell surface polarity complexes that appear to precede the initiation of TJ formation. We hypothesised that such an interaction would be important to coordinate the establishment of apical-basal polarity with cell proliferation through Hpo pathway regulation of cell density. Several interactions between Ocln and Hpo pathway were analysed in two cell line models with different growth characteristics, AsPc-1 and HPAFII (Figure 4.6, Figure 4.7).

In HPAFII cells, YAP and c-Yes co-localised extensively at the cell membrane and cytoplasm but not in the nucleus of HPAFII cells. Ocln and c-Yes co-localised at the cell membrane but these occurred to a lesser extent than c-Yes and YAP; Like Ocln and YAP distribution, these interactions were mostly confined to the cytoplasmic region. Ocln and YAP co-localised essentially at the external facing surfaces of these cell aggregates and Ocln and TEAD co-localisations appeared to be diffusely distributed throughout the cytoplasm. These results are consistent with previous demonstrations that c-Yes/Ocln and c-Yes/YAP interactions occur in epithelial cells (Chen et al., 2002b; Sudol, 1994) but demonstrate for the first time, potential interactions between Ocln and the Hpo signaling elements YAP and TEAD.

In AsPc-1 cells, the interactions between YAP and c-Yes occurred mostly in the cytoplasm and not so much in the nucleus whereas Ocln and c-Yes co-localised predominantly at the membrane similarly to that observed in HPAFII cells (Figure 4.7). YAP and Ocln interactions occurred predominantly at the plasma membrane and in the nucleus of AsPc-1 cells, different from that observed for HPAFII cells. Besides, as opposed to HPAFII cells, TEAD and Ocln interaction was almost non-existent and failed to show cytoplasmic co-localisations (Figure 4.7). Co-localisation of Ocln and YAP at the cell membrane of aggressive growing AsPc-1 cells was further confirmed through immunoprecipitation assay (Figure 4.8). Our search for such a sensor uncovered an interaction between YAP and Ocln, an integral membrane protein of the TJ complex that forms homotypic extracellular contacts (Furuse et al., 1993).

Pharmacological shifting of YAP from the nucleus to the cytoplasm, using the β-adrenergic receptor agonist dobutamine, increased the extent of Ocln expression at the cell surface in bi-cellular contact surfaces in both HPAFII and AsPc-1 cell lines (Figure 4.9 and Figure 4.16). Dobutamine also induced TEAD translocation from the nucleus and stabilisation of Ocln at the cell membrane of HPAFII cells (Figure 4.10). In AsPc-1, nuclear TEAD levels were reduced after dobutamine treatment (Figure 4.18). Liu-Chittenden, showed that disruption of the interaction between YAP and TEAD could block YAP transforming activity in HEK293 cells and out of a small molecule library screen were able to find three compounds able to inhibit YAP transcription (Liu-Chittenden et al., 2012); the compounds identified, all from the porphyrin family, were protoporphyrin IX, hematoporphyrin and verteporfin. Particularly, the compound Verteporfin (Visudyne®) is already used in the clinic and it was shown to inhibit the interaction between YAP and TEAD *in vitro* (Liu-Chittenden et al., 2012).

Nuclear c-Yes distribution increased progressively in CP and in PDAC tissue samples. This increase in c-Yes expression is consistent with previously observed changes in pancreatic cancer (Kubo et al., 2009) (Figure 4.11). Subsequently, Ocln and c-Yes interactions were monitored after dobutamine treatment. Dobutamine enhanced the distribution of Ocln at the plasma membrane in both cell types where increased interactions with c-Yes (Figure 4.12 and Figure 4.17) and decreased interactions with YAP in AsPc-1 cells (Figure 4.16) could be observed, consistent with early observations in HPAFII cells (Figure 4.9 and Figure 4.12).

Ocln regulated in this way was observed to be functional at cell-cell contacts as demonstrated by its ability to restrict Tric from bi-cellular contacts and by increasing epithelial barrier properties (Figure 4.13), findings consistent with previous studies examining Ocln function (Ikenouchi et al., 2005; Ikenouchi et al., 2008). Two striking differences between AsPc-1 and HPAFII cells were nuclear YAP and TEAD content (Figure 4.10 and Figure 4.18) along with altered CRB3-Ocln co-localisations (Figure 4.19). While dobutamine treatment failed to dramatically alter the CRB3-Ocln co-localisation observed in HPAFII cells, it did affect YAP and TEAD distribution (Figure 4.19 and Figure 4.10).

Dobutamine treatment also accelerated and enhanced epithelial cell apical-basal polarization (Figure 4.14). Particularly, dobutamine appeared to induce the formation of “rosette” structures indicative of luminal-like organization in HPAFII cells cultured in a 2-D system (Figure 4.15). Despite the fact that a luminal marker to confirm the formation of a single hollow lumen was not used in this study, this observation opens up the possibility of using this *in vitro* system as a powerful tool to study epithelial architecture and morphogenesis of the pancreas under close to physiological conditions. Culturing HPAFII cells in extracellular matrixes such as collagen I or matrigel, could potentially create a 3-D culture system in which a spherical epithelial monolayer with a central single hollow lumen would be formed. In essence, such specialised systems constitute a powerful tool that can

shed light on how cell polarity is regulated and how does it interact with the extracellular matrix. Additionally, 3-D culture of tumour cells recapitulates pancreatic cancer physiology and tumour microenvironment providing a more reliable and high-throughput screening platform for chemotherapeutic drugs.

The role of YAP in malignancy still remains controversial and accumulating evidence suggests that the dual roles as an oncogene and tumour suppressor gene in human oncogenesis appear to be tissue and cell context specific. It has become clear that the interplay between YAP binding to transcriptional factors and its interaction with different signaling pathways as well as the specific stimuli acting on a particular cell or tumour type, determine the dual function of YAP as an oncogene or as tumour suppressor gene. Several studies have linked several proliferation markers to YAP, yet it is still not clear if the expression of YAP in epithelial cancers induces tumourigenesis or if it contributes to a more favorable cellular environment for the development of tumours (Camargo et al., 2007; Zhao et al., 2007). Under normal conditions, YAP functions to maintain tissue homeostasis, however once it becomes deregulated it can contribute towards a malignant phenotype. Being a nuclear effector, YAP might cause genomic instability sustaining an environment that promotes cell proliferation and inhibits apoptosis.

Most epithelial cancers, including pancreatic cancer, that contain mutant K-Ras (Kanda et al., 2012; Morris et al., 2010) and have somatic mutations in the small *K-RAS* gene are generally associated with poor response to standard therapies (Ostrem et al., 2013). Despite the efforts in developing targeting therapies directed to oncogene-addicted tumour, nearly all tumour remissions are followed by tumour relapse and acquired resistance (Greten, 2014). PDAC cells have been reported to be able to survive in the absence of oncogenic K-Ras signaling and acquiring alternative mechanisms of resistance that sustain their continuous

growth (Greten, 2014). Recent studies reported that YAP can substitute for loss of K-Ras and YAP and the transcriptional factor TEAD-2 was suggested to be responsible to drive K-RasG^{12D} – independent tumour maintenance in a mouse model of PDAC (Kapoor et al., 2014). YAP has also been shown to be able to rescue cell viability in K-Ras-dependent cells upon suppression of K-Ras (Shao et al., 2014b). These studies have highlighted a novel mechanism of resistance to K-Ras inhibition through Ras signaling replacement by YAP (Kapoor et al., 2014). These findings have paved the way to identify small-molecule modulators of Hpo signaling as novel and exciting approaches for cancer therapy.

Identification of pathways or molecules that are required for tumour growth provides a new strategy that would overcome the drawbacks of conventional cancer treatments. The Hpo signaling pathway is an emerging growth control and tumour suppressor pathway portending the need for YAP inhibitors (Genevet and Tapon, 2011; Halder and Johnson, 2011; Stanger, 2012). The fact that altered Hpo signaling cascade appears to be tightly linked to tumour initiation and/or progression makes this pathway an attractive target for therapy. Nevertheless, part of the downstream effectors of the Hpo pathway are kinases that function as negative regulators by inhibiting YAP function, so targeting inhibitors to these constituents might result in YAP activation and consequently induction of cell proliferation (Stanger, 2012).

In particular, in this study, YAP seemed to act as an oncogene and dobutamine was introduced as a promising strategy to target pancreatic cancer more effectively. Dobutamine treatment was shown to lead to suppression of YAP localisation to the nucleus and hypothesised that Ocln re-expression in cancer cells functions in a fail-safe mechanism to induce cell death, which will be further discussed into more detail in the following chapter (Figure 4.19). It has been shown by different studies that re-expression of Ocln in cancer

rescues the malignant phenotype back to normal and these cells are then able to polarise again (Li and Mrsny, 2000). Thus strategies that re-express endogenous Ocln in cancer might open up new therapeutic modalities. However, since these results have been obtained in an *in vitro* system, conclusions for clinical application should be drawn prudently. *In vivo* studies were able to show that knock-down of YAP affects pancreatic cancer cell growth and YAP expression enables tumour growth even in the absence of K-Ras (Greten, 2014). Given the potential interest in targeting the Hpo pathway to allow for a redesign of novel pancreatic cancer therapies, there is a considerable value in testing dobutamine in a YAP knock-down mice model.

These findings have linked Hpo pathway to the actin cytoskeleton, cell junctions and cell polarity. Our studies have now addressed the identification of a potential extracellular cell density sensor that can fine-tune the suppression of cell proliferation. Ocln, the first integral membrane protein found to be associated with TJ structures, has the characteristics that suggest it to fulfill this cell density sensor role through a mechanism that involves its removal from the plasma membrane in the absence of cell-cell interactions.

Overall, our data suggest that Ocln is uniquely positioned at TJ structures to register the extent of a functional barrier for the coordination of cell density and apical-basal polarity (Figure 4.20). Importantly, this work suggested that Ocln is able to directly interact with YAP's co-activator, TEAD, in a manner that could suppress cell proliferation (discussed in more detail in Chapter 5). These studies also showed that treatment with dobutamine, currently approved to treat acute heart failure, can reactivate Ocln expression and cell surface distribution. Finally, the *in vitro* findings were correlated with the expression pattern of Ocln and Hpo signaling elements in human pancreas biopsies obtained from healthy individuals or individuals diagnosed with CP and PDAC (Cravo, et al., 2014, submitted). A

role for Ocln as a sensor for planar cell polarity that intersects with the Hpo pathway provides some potential explanations for the complex and poorly understood function(s) of this TJ protein. Previous studies have shown the TJ protein AMOT and Crumbs complex components to help sequester YAP to the TJ (Zhao et al., 2011). Our data now shows that Ocln, a TJ protein that is rapidly regulated at bi-cellular TJ structures (Furuse, 2010), is capable of relaying information relevant to the extent apical-basal polarization through YAP and TEAD to provide a potential sensor function for Hpo pathway regulation (Figure 4.20). In this way, Ocln interactions with YAP and TEAD could couple apical-basal polarity with lateral cell density to allow epithelial cells to calibrate their bias towards proliferation versus polarization (Figure 4.20).

Ocln interactions with Hippo Pathway Signaling

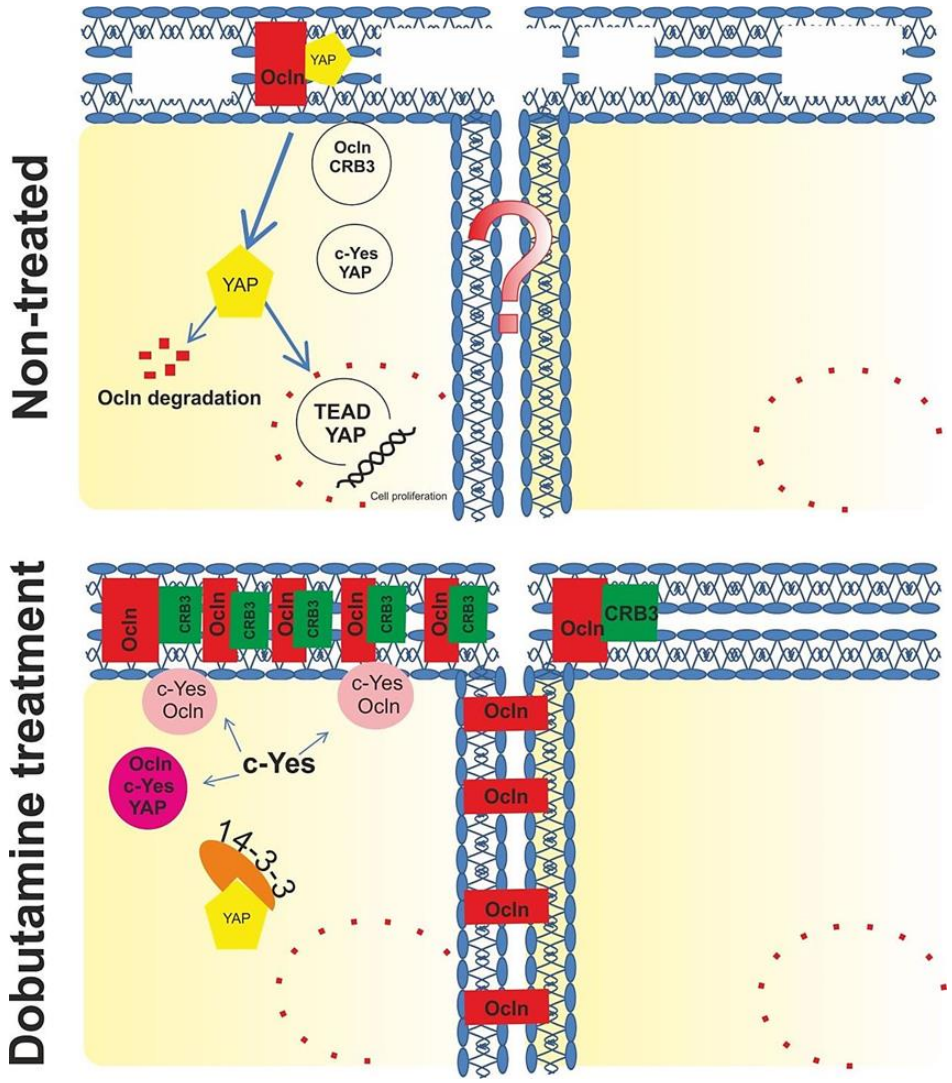

Figure 4.20 Schematic representation of dobutamine molecular actions in cancer cells

Chapter 5

5 Dobutamine – a potential Adjuvant Molecule in the Treatment of Pancreatic Cancer

5.1 Background

The Hpo pathway is activated by different upstream molecular signals including cell-cell contact and cell polarity molecules, adhesion and junction proteins and also GPCRs (Yu et al., 2012). In mammals, adrenergic activity modulates the rate of cell replication and differentiation (Slotkin et al., 1988); Catecholamines, activators of GPCRs, play a role in controlling cell replication and differentiation (Slotkin et al., 2000). Adrenergic gene knockouts have lethal effects (Zhou et al., 1995) and β-adrenergic signalling can regulate the function of several epithelial cancer cell types (Baker et al., 2011; Daly and McGrath, 2011). In adulthood adrenergic control terminates as cells exit mitosis and transition to the terminal differentiation stage. Some epithelial cancers and cancers of secretory cells, however, can re-express β-adrenergic receptors (MacEwan and Milligan, 1996; Mitra and Carraway, 1999). Epidemiologic studies associating stress levels with accelerated tumour progression have prompted studies involving β-adrenergic regulation in tumour biology (Antoni et al., 2006; Chida et al., 2008).

A hallmark of epithelial cancers is the loss of functional cell-cell contacts involving AJs and TJs (Martin et al., 2010). In this regard, stable TJ structures have a crucial role in suppressing cell proliferation and in priming cells for apoptotic induction in epithelial cells (Balda and Matter, 2009). The function of Ocln has been poorly understood since its identification as the first integral membrane protein of the TJ (Furuse et al., 1993). Subsequently, it was demonstrated that TJ structures could form in the absence of Ocln (Saitou et al., 2000), but studies have shown that its presence can modulate certain aspects of epithelial barrier function (Yu et al., 2005). The cytoplasmic C-terminal tail of Ocln has been shown to bind directly to PKCζ (Nusrat et al., 2005). This serine/threonine kinase also functions as a cell polarity regulator through its interactions with PAR3 and PAR6

(Liu et al., 2004). Additionally, PKCζ can associate with KIBRA, an upstream regulator of the Hpo pathway (Yoshihama et al., 2012). Since the TGF-β-SMAD pathway couples with Crumbs complex to provide Hpo-dependent control of cell density (Varelas et al., 2010), it is interesting how TGF-β signalling and Ocln function seem to intersect (Barrios-Rodiles et al., 2005).

The Rho Kinase (ROCK) previously shown to regulate epithelial paracellular permeability (Walsh et al., 2001), has been suggested to have a critical role in TJ assembly (Terry et al., 2010; Walsh et al., 2001); however Rho regulation of TJ organization has not been reported to be directly associated with Ocln. As shown in the previous chapter, Chapter 4, dobutamine, a currently approved drug in clinic to treat acute heart failure, induces the re-establishment of functional cell-cell contacts that have been lost or reduced in epithelial cancers. Several studies have suggested the potential adjuvant effect of dobutamine in the treatment of several epithelial cancers (Bao et al., 2011; Sun et al., 2014a); no studies have yet reported the potential benefit in using this β-agonist in the treatment of pancreatic cancer.

In this chapter, the actions of dobutamine on pancreatic cancer cell lines *in vitro* to identify possible applications for the treatment of PDAC, were evaluated. The actions of dobutamine on pancreatic cancer cell viability, proliferation and apoptotic induction were examined alone and in combination with gemcitabine as a potential adjuvant chemotherapeutic approach. Finally, aiming at increasing the selectivity and reducing off-target interactions, PepT1 expressing cells were treated with a PepT1 targeted gemcitabine chimera combined with dobutamine and cell proliferation rates were assessed.

5.2 Materials and methods

5.2.1 Cell viability and proliferation assays

AsPc-1 (passage 15) and HPAFII (passage 10) cells were seeded in 96-well plates at a cell density of 500 and 1000 cells per well respectively and allowed to attach. Following this, cells were supplemented with fresh medium containing either dobutamine or gemcitabine; for β-adrenergic mediation, cells were treated either with dobutamine on its own or in combination with the antagonists: atenolol (600 nM – Santa Cruz) and propranolol (12 nM - (Briley et al., 1980)). After 48 h incubation, in order to assess cell proliferation or cell viability, either CyQuant™ or MTT solution respectively, were added to each well as outlined in sections 2.7 and 2.8, Chapter 2. Each assay was done in triplicate in independent experiments.

5.2.2 Ocln knock-down

A pool of 4 individual siRNA (Accell Human OCLN, siRNA-SMARTpool, Thermo Scientific) targeting different sequences in the Ocln gene was used (Table 2.4, Chapter 2). For proliferation assays, HPAFII (passage 15) and AsPc-1 (passage 12) were grown on a 96 well plate to about 70 % confluency at a cell density of 1000 and 500 cells per well, respectively. For paracellular studies, HPAFII cells (passage 17) were seeded onto the apical chamber of polyester filters, as outlined in section 2.3.1, Chapter 2. For both proliferation and paracellular assessment studies, cells were treated with siRNA in parallel with a scrambled control siRNA, as described in section 2.11.5. After removing the treatment media, a cell proliferation assay was carried out following the procedure described

in section 2.7, Chapter 2. For protein levels, cells were harvested and run on a gel for Western Blot analysis, as mentioned in sections 2.4 and 2.6 respectively, Chapter 2.

5.2.3 TER measurement and paracellular permeability studies

For polarization experiments, HPAFII cells (passage 15) were seeded onto the apical chamber of polyester filters, as outlined in section 2.3.1, Chapter 2. TER measurements were performed on a daily basis (section 2.3.2, Chapter 2) and on day 7, cells were treated with 20 μM ROCK inhibitor or 50 μM PKCζ pseudosubstrate (PKCζ-PS) either alone or in combination with 20 μM dobutamine (Cerruti et al., 2013). At day 11, paracellular studies were carried out as outlined in section 2.3.3, Chapter 2. For TGF-β experiment, HPAFII cells (passage 21) were allowed to grow for 10 days. On day 11, TGF-β was applied apically either alone or in combination with 20 μM dobutamine. After 48 h, paracellular permeability was assessed as described in section 2.3.3, Chapter 2.

5.2.4 Immunostaining

At the end of paracellular studies, filters were carefully cut and cells were blocked and permeabilised. Following this, cells were mounted on slides and examined through confocal microscopy as described in section 2.9, Chapter 2.

5.2.5 FACS

AsPc-1 (passage 20) and HPAFII (passage 6) were seeded in T 25 cell culture flasks till approximately 70 % confluency. Dobutamine and gemcitabine, either alone or in

combination, were added to each flask in a total volume of 5 mL of cell media and left incubating for 48 h. Subsequently, cells were trypsinized and centrifuged as outlined in section 2.1, Chapter 2; cell pellet was re-suspended in 100 µL of annexin-V-FLUOS and analysed through FACS as referred in section 2.12, Chapter 2. Controls for this experiment can be seen in Appendix (Figure 8.1 and 8.2, Chapter 8).

5.2.6 Annexin V/PI cell staining

AsPc-1 (passage 22) and HPAFII (passage 7) were seeded in plastic micro-dishes with high glass bottom (Thistle Scientific Ltd) till reaching approximately 70 % confluency. Dobutamine or gemcitabine, both alone or in combination, were added to each flask in a total volume of 300 µL cell media and left incubating for 48 h. As a positive control for apoptosis, cells were treated with staurosporine, a well-established apoptotic cell inducer (Chae et al., 2000). As a positive control for necrosis, cells were treated with 1 % Triton X-100, a reported necrotic inducer (Goto et al., 2009). Subsequently, cells were stained for annexin V/PI and analysed through confocal microscopy as outlined in Chapter 2, sections 2.12.1.1 and 2.9 respectively.

5.2.7 ELISA: nucleosome detection

AsPc-1 (passage 19) and HPAFII (passage 10) were seeded in 96 well plates at a cell density of 500 and 1000 cells per well respectively. Dobutamine or gemcitabine, both alone or in combination, were added to each well in a total volume of 100 µL. After 48 h incubation, a photometric enzyme immunoassay for *in vitro* detection of cytoplasmic histone-associated DNA fragments as referred in section 2.12.1.2, Chapter 2, was carried out. As a positive control for apoptosis, cells were treated with staurosporine for 4 h (Chae et

al., 2000). As a positive control for cell necrosis, cells were treated with cell media supplemented with 1 % Triton X-100 (Goto et al., 2009).

5.3 Results

5.3.1 Dobutamine suppresses cell proliferation in vitro

While the barrier properties of TJ network have been extensively studied, it is now becoming more evident the complexity of TJ as a platform that integrates multiple signals involved in cell polarity and tissue homeostasis comprising cell differentiation, proliferation and migration (Farkas et al., 2012). Induction of cell proliferation and suppression of apoptotic pathways are the two critical features of Hpo pathway deregulation. As previously mentioned, YAP binds to the TEAD family of transcription factors which are involved in the regulation of CTGF which in turn drives cell proliferation . Since the transcriptional co-activator YAP/TEAD is known to affect cell proliferation rates (Camargo et al., 2007) and YAP is involved in the regulation of proliferative and anti-apoptotic pathways (Bao et al., 2011; Strano et al., 2005), the goal was to examine the effects of dobutamine treatment on cell proliferation (Figure 5.1). Under normal conditions, HPAFII cells were grown from a seeding density of ~10 % confluence, reaching ~50 % confluence by 24 h and ~70 % confluence by 48 h; dobutamine treatment of HPAFII cells, seeded at a low cell density, significantly reduced their proliferation in a dose-dependent manner when examined at both 24 h and 48 h (Figure 5.1-A). A comparable dobutamine-induced reduction in proliferation rates was observed for AsPc-1 cells at 24 h but significantly more pronounced at 48 h (Figure 5.1 –B).

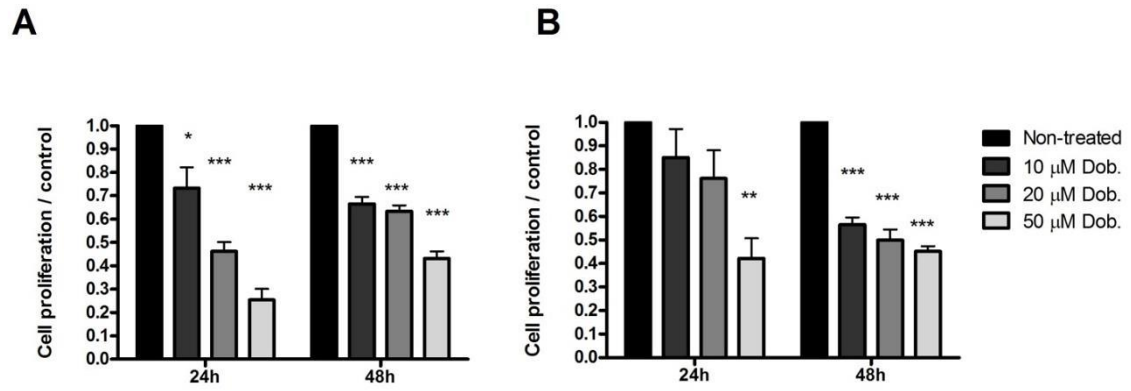

Figure 5.1 The β-agonist, dobutamine, suppresses cell proliferation in two pancreatic cancer cell lines *in vitro*. Cell proliferation of HPAF II cells **(A)** and AsPc-1 cells **(B)** exposed to 10, 20 and 50 μM dobutamine over 24 h and 48 h; values are expressed as mean ± SEM from 3 independent experiments; * $p < 0.05$; ** $p< 0.01$; *** $p<0.001$ compared to non-treated cells.

After confirming that 48 h was the optimal exposure time to observe a pronounced anti-proliferative effect, we intended to characterize HPAFII and AsPc-1 pharmacological responses in terms of cell proliferation and viability mediated through dobutamine treatment. Through live cell fluorescent labelling and MTT assay, dobutamine half maximal effective concentration (EC_{50}) for reducing cell proliferation and viability was determined. As this data shows, dobutamine affected differently cell viability and cell proliferation in these two pancreatic cell line models. The concentration needed to decrease cell viability after 48 h was much higher in AsPc-1 compared to HPAFII cells. Conversely, AsPc-1 cells showed a slightly reduced EC_{50} for proliferation compared to the slow growing epithelial cell line model, HPAFII. From this analysis, it can be concluded that dobutamine decreased cell viability more markedly in HPAFII than in AsPc-1 cells and decreased cell proliferation in a slightly more pronounced manner in AsPc-1 cells in comparison to HPAFII cells (Figure 5.2).

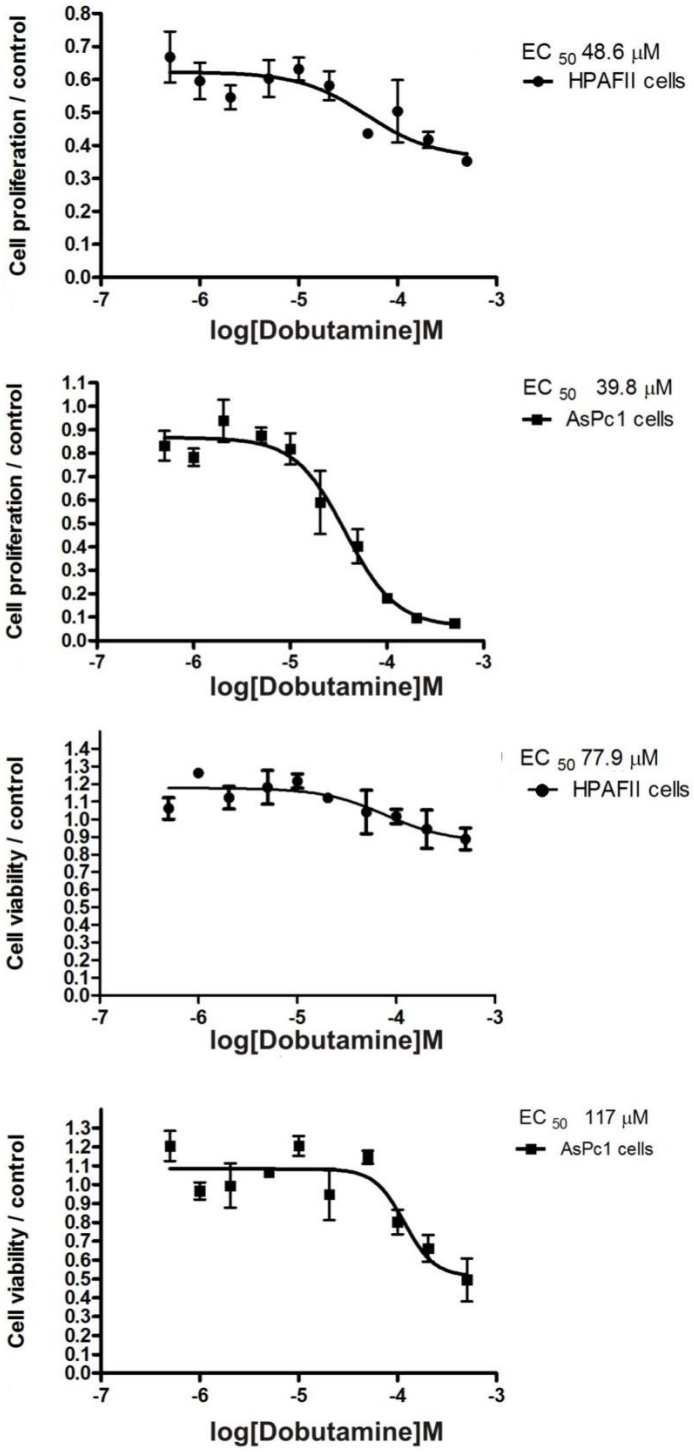

Figure 5.2 Dobutamine decreases cell viability more strikingly in HPAFII in comparison to AsPc-1 cells and decreases cell proliferation in a slightly more pronounced manner in AsPc-1 cells compared to HPAFII cells. Sigmoidal dose response curves showing the EC_{50} values obtained for cell proliferation and cell viability studies during a 48 h time course. Values are expressed as mean ± SEM from 3 independent experiments.

5.3.2 The suppression of cell proliferation via dobutamine is β-adrenoreceptors mediated

Being primarily a β_1-adrenergic agonist (Rang, 2003), it was investigated if the cellular responses induced by dobutamine were mediated through adrenergic β-receptors. Firstly, the expression profile of β-adrenergic receptors in the pancreatic cancer cell lines: AsPc-1 and HPAFII, was assessed through the database Oncomine (http://www.oncomine.org). AsPc-1 and HPAFII cells are reported to express both β_2 adrenergic receptors whereas only AsPc-1 cell lines express the β_1 sub-type (Figure 8.7-8.10, Chapter 8). In order to assess this, cell proliferation assays were carried out and in parallel bright field images were taken; In order to fully address if dobutamine actions could be antagonised by either propranolol or atenolol, AsPc1 and HPAFII cells were exposed to a much higher dobutamine concentration than the obtained EC_{50} for these cell lines. Cells were treated with dobutamine alone and in the presence of the non-selective and the β_1- selective receptor antagonist, propranolol (Black et al., 1964) and atenolol respectively (Brown et al., 1976). As mentioned before, dobutamine acts primarily on β_1 receptors and atenolol is a selective β_1 receptor antagonist (Brown et al., 1976). As shown in Figure 5.3-A, 500 μM dobutamine completely inhibited cell proliferation in HPAFII and AsPc-1 cells; however the β_1 selective antagonist atenolol, blocked dobutamine-induced suppression of proliferation in AsPc-1 but not in HPAFII cells (Figure 5.3-B). This is consistent with the fact that AsPc-1 cells are reported to express β_1 adrenoreceptor sub-type, whereas HPAFII only express β_2 (Chapter 8, Figure 8.7-8.10). Treating these cells with the antagonists on their own, produced a comparable effect (Figure 8.6, Chapter 8). Atenolol decreased cell proliferation in AsPc-1 whereas in HPAFII it did not produce any effect whereas propranolol produced the opposite effect by reducing proliferation levels in HPAFII and not in AsPc-1 cells (Figure 8.6, Chapter 8). Conversely, propranolol did not abrogate the anti-proliferative effect of dobutamine as shown by the phenotype presented by AsPc-1 cells and cell proliferation assays. These data suggests

that, opposite to HPAFII cells, the anti-proliferative effect of dobutamine in AsPc-1 appears to be β-receptor mediated, as the selective antagonist atenolol blocked the actions of dobutamine. Propranolol also blocked dobutamine-induced effects, however being a non-selective antagonist it cannot be ruled out that the reduction in cell proliferation observed in both cells lines is mediated through β-adrenergic receptors. Nevertheless, these results are consistent with the study carried out by Bao and co-authors, in which propranolol supressed the dobutamine induced phosphorylation and translocation of YAP, whereas a non-specific α adrenergic receptor inhibitor, phentolamine had no effect (Bao et al., 2011).

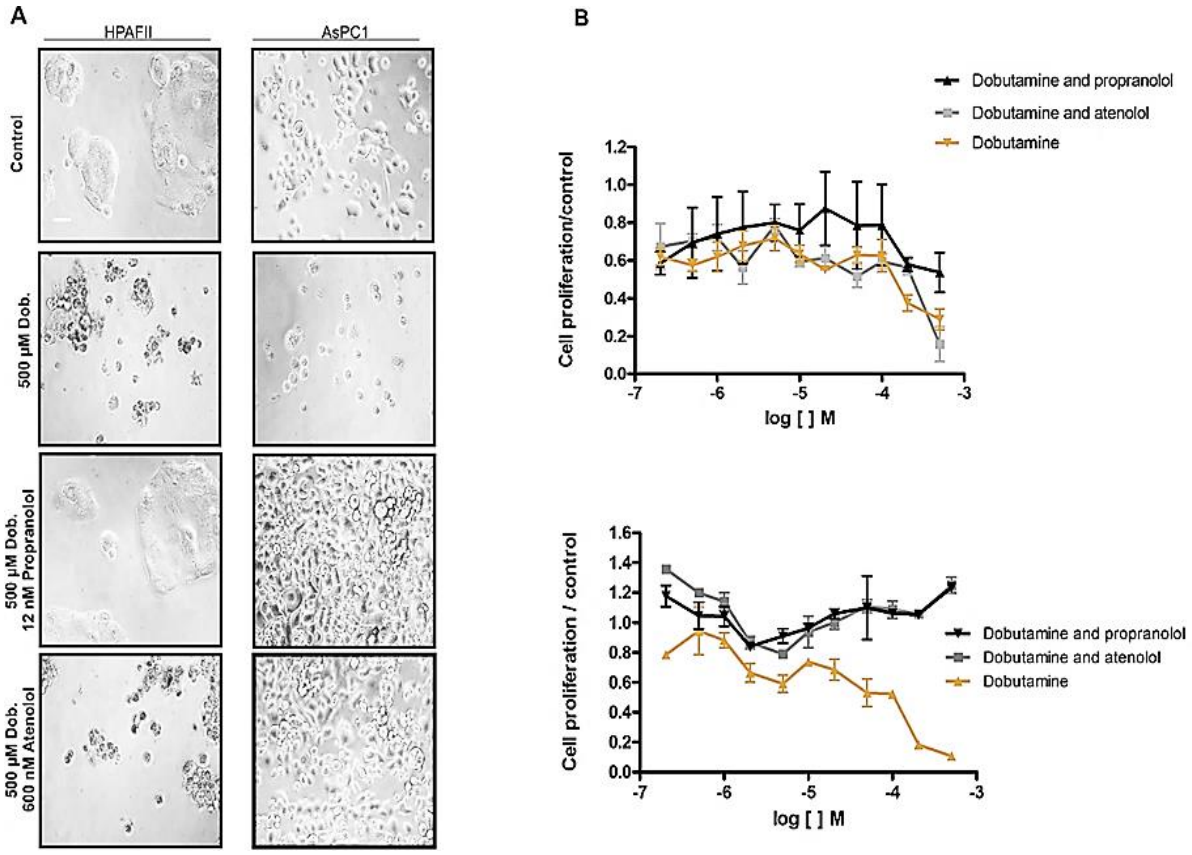

Figure 5.3 Decrease of cell proliferation induced by dobutamine is likely to be β-adrenergic dependent. (A) Bright field images of HPAFII and AsPc-1 cells exposed to 20 µM dobutamine, either alone or in combination with the antagonists 12 nM propranolol and 600 nM atenolol during 48 h; scale bar 50 µm; **(B)** Cell proliferation assay after HPAFII (top panel) and AsPc-1 (bottom panel) cells were exposed to 500 µM dobutamine, either alone or in combination with the antagonists 12 nM propranolol and 600 nM atenolol during 48 h. Values are expressed as mean ± SEM from 3 independent experiments.

5.3.3 Dobutamine inhibition of HPAFII and AsPc-1 cell proliferation involves Ocln

Suppression of cell proliferation and the activation of cell death mechanisms have been associated with the presence of stable and functional tight junction structures as well as a functional apical-basal polarity complex (Farkas et al., 2012). The presence of stable Ocln structures at the cell membrane after dobutamine treatment, as previously shown in Chapter 4: Figure 4.9, would shift the nuclear distribution of the co-activator factors YAP and TEAD to the cytoplasm, hence leading to a reduction in cell proliferation. Since the transcriptional co-activator YAP/TEAD is known to affect cell proliferation rates (Chen et al., 2010), we wished to determine if the dobutamine-induced effects on cell proliferation would involve Ocln regulation. To assess this, Ocln expression was transiently knocked down through siRNA and cell proliferation was assessed through a live cell fluorescent label. Knock-down of Ocln expression blocked the dobutamine-induced decrease in proliferation observed in both HPAFII and AsPc-1 cells (Figure 5.4). Interestingly, siRNA-mediated Ocln knock-down did not, by itself, result in a change in cell proliferation rates; it should be noted that the siRNA was added at the beginning of these experiments and it would have taken several hours and probably repeatedly dosing to affect significantly Ocln levels. These studies suggest that dobutamine, through events that involve Ocln, can reduce proliferation rates in these two pancreatic cancer cell lines with different capacities to undergo apical-basal polarization.

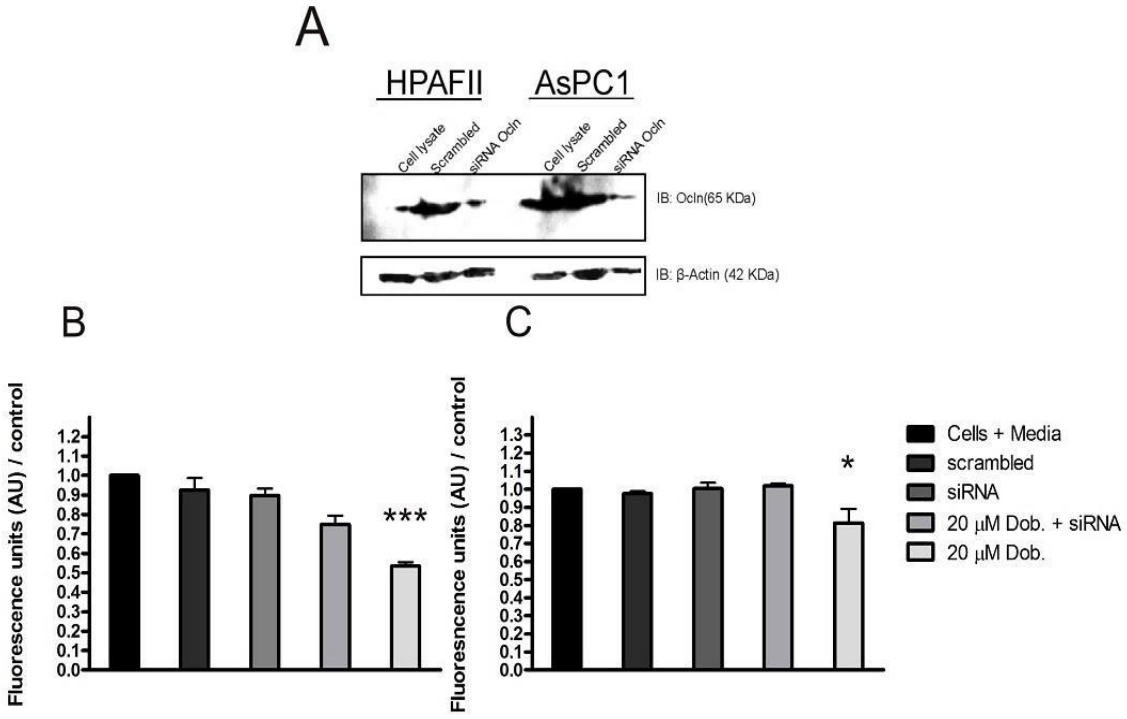

Figure 5.4 Ocln stabilization at the cell membrane induced by dobutamine treatment reduces cell proliferation. **(A)** Immunoblot showing Ocln knock-down; HPAFII **(B)** and AsPc-1 **(C)** cells treated with siRNA targeting Ocln or a scrambled, control siRNA at the same time as dobutamine was added to initiate the time course of the study. Cell proliferation was determined using a live cell fluorescent label. Values are mean of 3 independent experiments compared to siRNA treated levels ± SEM; * $p < 0.05$; *** $p < 0.001$.

5.3.4 Dobutamine treatment reduces size-selectivity of tight junction paracellular permeability

After confirming the involvement of Ocln in reducing cell proliferation, we wished to examine if after dobutamine treatment Ocln would be functionally active in limiting the diffusion of a fluorescently molecular probe across the TJ network. HPAFII cells were seeded on trans-well filters and 10 and 20 μM dobutamine was applied apically. As shown by Figure 5.5-A, the dobutamine-induced decrease in paracellular permeability was statistically significant compared to non-treated monolayers of HPAFII. To exclude the possibility of more than one HPAFII mono-layer growing on top of each other, z-stacks were taken and

confirmed single mono layers of HPAFII cells was being analysed (Figure 5.5 – B). This data suggests that Ocln stabilization at the cell membrane induced by dobutamine treatment plays an important role in TJ integrity.

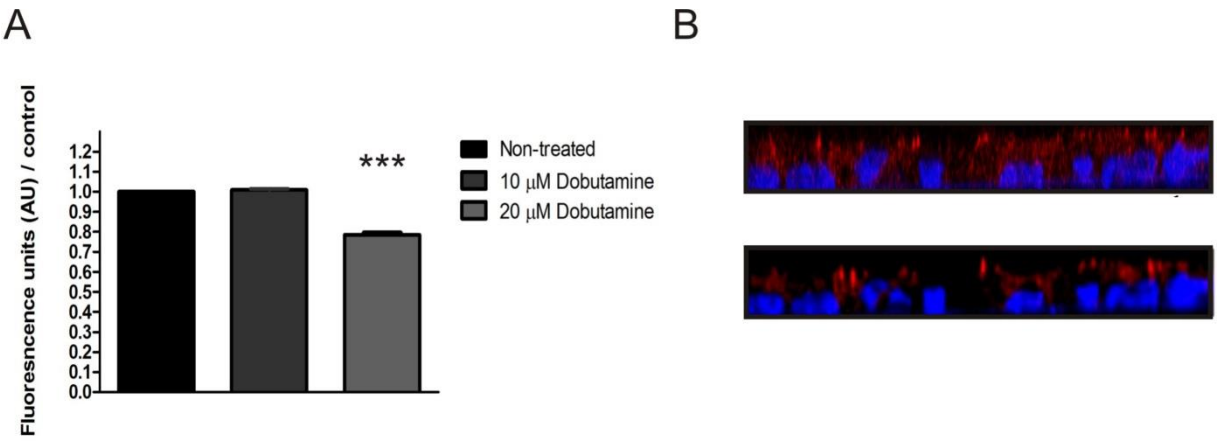

Figure 5.5 Dobutamine reduces paracellular permeability in HPAFII monolayers. (A) Tight junction paracellular permeability was assessed using fluorescently labelled 4 kDa Dextran; **(B)** Z-stacks of HPAFII monolayers either non-treated (top panel) or 20 μM dobutamine treated (bottom panel). Values are expressed as mean ± SEM from 3 independent experiments; ***p< 0.001.

5.3.5 Dobutamine rescues the effect of cell polarity disruptors through Ocln reactivation

Tissue disorganization and disturbance of cell polarity are both a hallmark of cancer accounting for more than 90 % of epithelial cancers. Three protein complexes are involved in establishing and maintaining apical-basal polarity: Crumbs, PAR, and Scribble (Bryant and Mostov, 2008). The core polarity complex is located at the cytoplasmic surface of the plasma membrane and is composed of three proteins: an atypical protein kinase C, PAR-3, and PAR-6 (Paris et al., 2008). Importantly, the hydrophobic surface of the cytoplasmic coiled-coil domain of Ocln can directly interact with at least three different proteins known to regulate TJ organization/disorganization and cell polarity: PKCζ, the regulatory subunit of phosphoinositol-3-kinase (RS-PI3K), and the proto-oncogene c-yes (Nusrat et al., 2000). PKCζ and Rho kinase have both been demonstrated to play critical roles in TJ organization

and stabilization (Etienne-Manneville and Hall, 2002; Suzuki et al., 2001) and more specifically, PKCζ has been reported to interact directly with Ocln (Nusrat et al., 2000). Inhibition of PKCζ leads to disassembly of cell polarity, thus we wished to test its role in processes involving dobutamine mediated TJ stabilization through pharmacological suppression using the PKCζ-PS peptide inhibitor (Lim et al., 2008). Firstly, phenotypic features of HPAFII in the presence of PKCζ-PS were examined either alone or in combination with the β-agonist dobutamine. HPAFII clusters of cells shrank considerably in the presence of the inhibitor and when dobutamine was added to the system, a more epithelial-like appearance could be observed, albeit these changes were very subtle (Figure 5.6 – A). To check for epithelial integrity, TER measurements were taken throughout the duration of the study. At day 7 an unexpected drop in TERs could be observed in cells treated with PKCζ-PS in combination with dobutamine, whereas cells that were treated with the β-agonist alone showed a progressive increase in TERs (Figure 5.6 – B). Despite the fact that an increase in Ocln production in cells treated simultaneously with dobutamine and PKCζ-PS inhibitor was observed, Ocln distribution was not observed at the cell surface (Figure 5.6 - C).

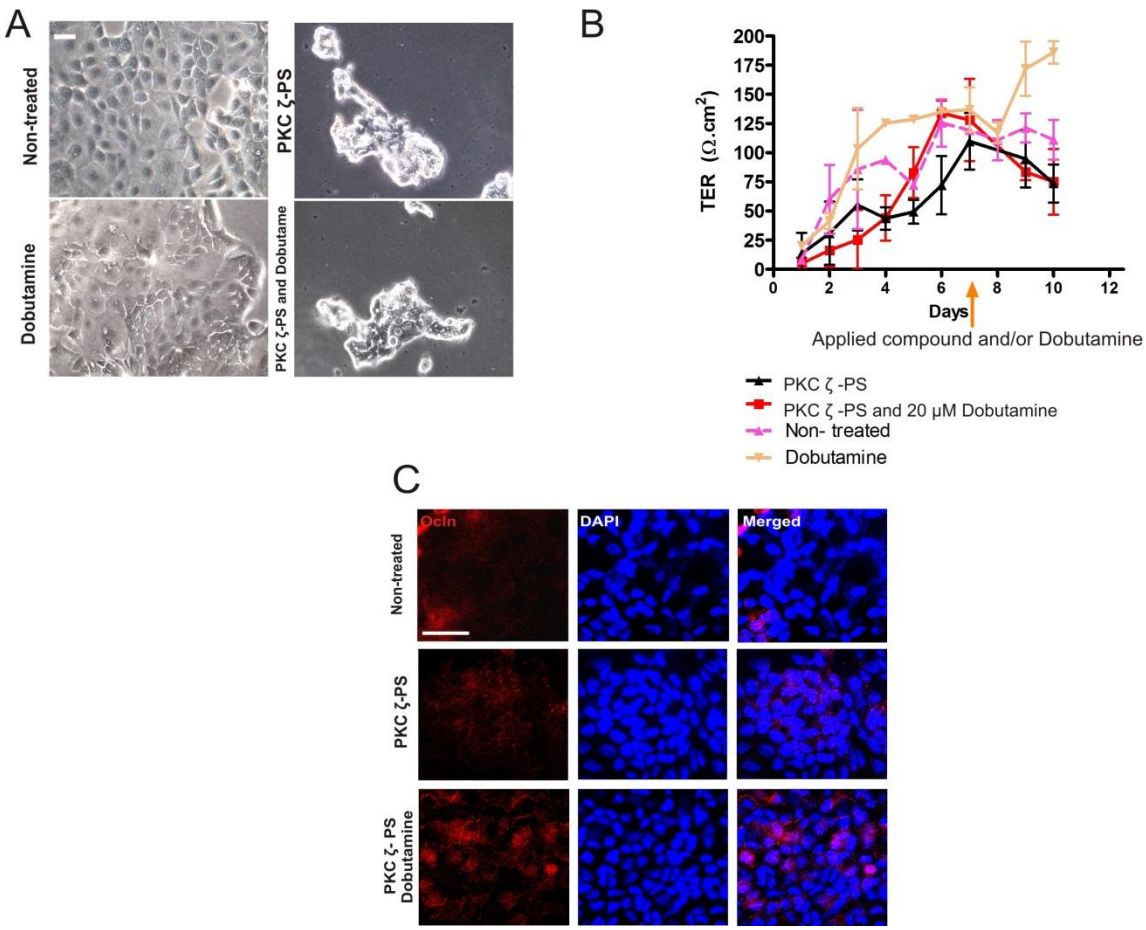

Figure 5.6 Dobutamine does not rescue the disruption of cell polarity induced by PKCζ-PS. (A) Bright field images of HPAFII cells seeded on 6 well plates after being treated with 50 μM of PKCζ-PS or 20 μM dobutamine, either alone or in combination compared to non-treated cells; scale bar, 50 μm; **(B)** TER measurements in HPAFII confluent monolayers seeded in trans-well filters - 50 μM of PKCζ inhibitor or 20 μM dobutamine, either alone or in combination, was added to the apical chamber on day 7; Values are expressed as mean ± SEM from 3 independent experiments; **(C)** Confocal microscopy images of HPAFII cells seeded on trans-well filters, after being exposed to PKCζ-PS either alone or in combination with dobutamine compared to non-treated cells; Scale bar, 20 μm.

Following this, other inhibitors of TJ function were tested to examine if dobutamine could reverse the disruption of cell polarity. ROCK posed as a good candidate to test as Rho GTPases play a crucial role in controlling cell adhesion and migration, actin cytoskeleton organization, microtubule dynamics, gene expression and vesicle transport (Etienne-Manneville and Hall, 2002). There are two Rho-Kinase members, Rho-Kinase α also known

as ROCK2 or ROKα and Rho-kinase β also called ROCK1 or ROKβ (Amano et al., 2010). Y-27632 inhibitor is a cell-permeable and selective inhibitor of ROCK1 activity (Ishizaki et al., 1997; Uehata et al., 1997). The Y-27632 inhibits ROCK by competing with ATP at the catalytic site. Inhibition of ROCK presumably disrupts actin-myosin contractility to induce a disorganization of cortical tension elements (Ishizaki et al., 2000; Matthews et al., 2006). Importantly, ROCK inhibition via Y-27632 can shift nuclear localisation of YAP in neuron cells (Sun et al., 2014b). In endothelial cells, dominant-negative ROCK constructs or ROCK inhibitors have been reported to decrease permeability suggesting that the activation of ROCK proteins lead to loss of TJ architecture (Wojciak-Stothard and Ridley, 2002). In epithelial cells, however, the compound Y-27632, has been shown to increase permeability. These controversial studies suggest that there are differences in cell signalling cascades linked to ROCK signalling in endothelial and epithelial cells (Walsh et al., 2001). To test whether reactivation of Ocln expression at the cell surface would rescue the phenotype induced by Y-27632, cells were treated in parallel with dobutamine and the ROCK1 inhibitor. After adding the ROCK inhibitor, TERs dropped in HPAFII cells which are consistent with several studies reporting the crucial role for ROCK in TJ assembly (Terry et al., 2010; Walsh et al., 2001). In cells treated simultaneously with dobutamine and the ROCK inhibitor, TERs decreased but in a less pronounced manner. Even though dobutamine was not able to restore completely the TERs levels back to the baseline (cells treated with dobutamine), at day 9 there was a significant difference of ~70 % in the TERs between cells treated simultaneously with dobutamine and the inhibitor compared to cells treated with the compound Y-27632 only (Figure 5.7-A). Although Ocln has been shown not to have a preponderant role in TJ formation (Saitou et al., 2000), it has been shown to have an important role in the regulation of paracellular barrier function (Saitou et al., 1998). It was hypothesised that the partial phenotype recovery induced by the compound Y-27632 administered together with dobutamine, was mediated through re-expression of Ocln at the

cell surface. To test this, Ocln expression was reduced through siRNA and the paracellular permeability of fluorescently labelled dextran was assessed in confluent epithelial monolayers of HPAFII. Cells treated with Ocln siRNA only presented considerably leaky epithelium which is consistent with previous studies (Balda et al., 2000; Bamforth et al., 1999); Knock-down of Ocln blocked the dobutamine-induced Ocln stabilisation at the cell membrane in HPAFII cells (Figure 5.7-B, Figure 8.3, Chapter 8). Conversely, dobutamine as expected, significantly decreased the permeability in HPAFII by ~ 60 % compared to non-treated monolayers; in cells that were exposed to the ROCK inhibitor, paracellular permeability was significantly enhanced suggesting the disruption of the epithelial barrier (Figure 5.7-B). After cells were treated simultaneously with siRNA and Y-27632, paracellular permeability had an increase of ~ 40 % compared to non-treated levels and when these cells were treated simultaneously with dobutamine, permeability levels decreased in a statistically non-significant manner (Figure 5.7-B). Walsh and co-workers suggested that ROCK inhibition and increase in paracellular permeability was not associated with redistribution of TJ and AJ proteins (Walsh et al., 2001) . Here it is proposed that through re-expression of Ocln at the cell surface induced by dobutamine, the molecular effects on TJ disruption induced by ROCK inhibition can be partially recovered. The addition of dobutamine in the presence of Ocln siRNA had no effect when added simultaneously with the ROCK inhibitor. A study suggested that knock-down of Ocln in MDCK cell lines did not affect the fence function of cells (Yu et al., 2005), however in this study we reported the decrease in paracellular permeability as Ocln function gets suppressed, These observations are consistent with our previous data in which it was showed that dobutamine reduced proliferation rates in two pancreatic cancer cell lines through events that involve Ocln (Figure 5.1 and 5.4). Because ROCK inhibition enhanced paracellular permeability and this biological effect appeared to be mediated through Ocln, we examined the distribution of this TJ marker by immunofluorescence labelling. Subsequently, we wanted to check Ocln

distribution after cells were treated with the ROCK inhibitor alone or with dobutamine (Figure 5.7-C). Ocln was detected at the cell surface of cells treated with dobutamine and Y-27632 whereas in the cells treated with the inhibitor only, Ocln distribution was restricted to the cytoplasm and cell nuclei (Figure 5.7-C). Walsh reported no changes in TJ distribution were detectable after ROCK inhibition (Walsh et al., 2001). In our cell model, however, phenotypic changes were observed; confluent cultures of HPAFII grown on plastic and exposed to the ROCK inhibitor presented with a fibroblastic-like appearance whereas cells treated with dobutamine and Y-27632 demonstrated subtle changes in phenotype consistent with an increase in the extent of cell-cell contacts mediated through dobutamine exposure (Figure 5.7-D). These data are consistent with work carried out by Ishizaki and co-workers in which Swiss 3T3 cells that were incubated with Y-27632 showed disruption of actin stress fibres (Ishizaki et al., 2000).

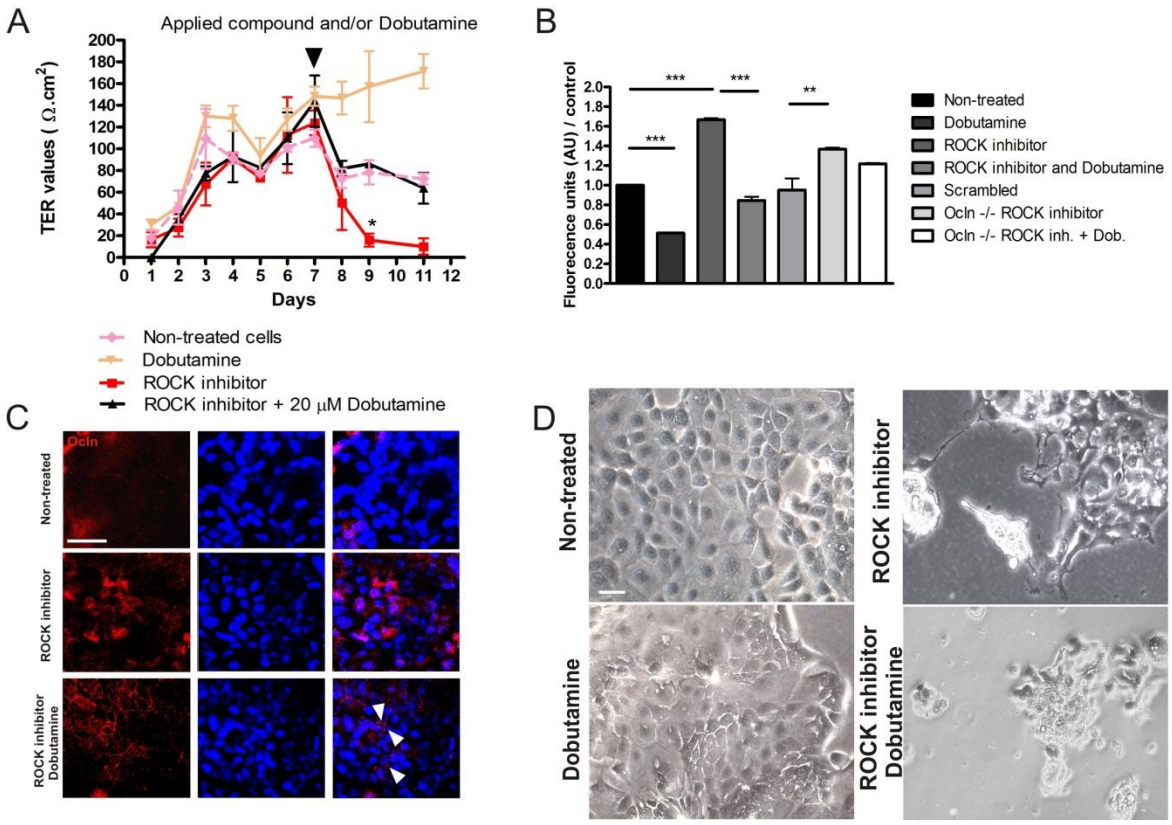

Figure 5.7 Dobutamine rescues the disruption of cell polarity induced by the ROCK inhibitor Y-27632; (A) TER measurements in HPAFII confluent monolayers seeded in trans-well filters with 20 μM of Y-27632 or 20 μM dobutamine, alone or in combination, being added to the apical chamber on day 7. Values are expressed as mean ± SEM from 3 independent experiments; * $p<0.05$; **(B)** Paracellular permeability of HPAFII cells cultured on trans-well filters, using 4 kDa fluorescently labeled FITC-dextran - 20 μM of Y-27632 or 20 μM of dobutamine, alone or in combination that were added to the apical chamber on day 11; afterwards fluorescence levels were measured in the receiver outer chamber; Values are expressed as mean ± SEM from 3 independent experiments, ** $p<0.01$, ***$p< 0.001$; **(C)** Confocal microscopy images of HPAFII cells seeded on trans-well filters, after being exposed to Y-27632 alone or in combination with dobutamine compared to non-treated cells; Scale bar, 20 μm; **(D)** Bright field images of HPAFII cells seeded on 6 well plates after being treated with 20 μM of Y-27632 or 20 μM dobutamine, alone or in combination compared to non-treated cells; scale bar, 50 μm.

5.3.6 Dobutamine is able to rescue the disruption of cell polarity induced by TGF-β

TGF-β is a cytokine involved in the modulation of cell differentiation, proliferation, migration, adhesion and apoptosis and contributes to the formation of the extracellular matrix (ECM). Being a pleiotropic molecule, TGF-β activity has a tight biological regulation (Kamaraju and Roberts, 2005). TGF-β has been shown to be a potent inhibitor of pancreatic acinar and ductal cell proliferation *in vitro* (Sanvito et al., 1994) and genetic mutations in pancreatic cancer may lead to loss of TGF-β responsiveness contributing therefore to tumour progression (Villanueva et al., 1998). In cancer, TGF-β has a dual role (Wakefield and Roberts, 2002); in the early stage of tumourigenesis, cells are still sensitive and responsive to the growth inhibitory effect of TGF-β thus acting as a tumour suppressor; in the later stages, though, it acts as a pro-metastatic agent as cells have escaped from its growth inhibitory effects (Nicolas and Hill, 2003). Nevertheless, certain TGF-β signalling pathways remain functional in these cells (Wakefield and Roberts, 2002). SMADs are the intracellular effectors of TGF-β (Derynck et al., 1996; Itoh et al., 2000). In some cell types, TGF-β-dependent SMAD signalling intersects with Ras/MAPK/MEK signalling pathway, in others it has been shown to be SMAD-independent utilizing instead the RhoA and the PI-3 kinase pathways (Bhowmick et al., 2001). The Rho/ROCK pathway has been reported to be crucial in mediating the growth inhibitory effects of TGF-β in MCF-10A breast cancer cell line (O'Mahony et al., 1998). After showing that loss of TER resulting from ROCK1 inhibition could be partially recovered with dobutamine treatment, we wanted to examine the contribution of TGF-β on its own and in combination with the β-agonist to TJ integrity (Figure 5.8). To test this, the rate of 4 kDa fluorescent dextran as a marker for paracellular flux was evaluated (Matter and Balda, 2003). Tight junction permeability in cells treated with TGF-β was higher compared to non-treated cells, although at 20 nM no significant changes between treated and non-treated cells were observed. At 40 nM of TGF-β treatment, tight

junction permeability decreased again. Dobutamine treatment however, in combination with TGF-β, diminished paracellular permeability in a statistically significant manner. Despite the dose response in paracellular permeability reduction at 5, 10 and 20 nM of TGF-β, we failed to observe a significant increase in the TER values. At the time this experiment was carried out, HPAFII cells were at a higher passage number (passage 21) than those used in previous studies and we have observed, surprisingly, that HPAFII cells start to lose their epithelial junctions after they have been cultured and passaged for a long time. When grown on plastic, these higher passage HPAFII cells formed small clusters in which lateral cell-cell contacts could be detected, but when cultured on filters the highest TER values obtained were around 250 Ω.cm^2 (data not shown).

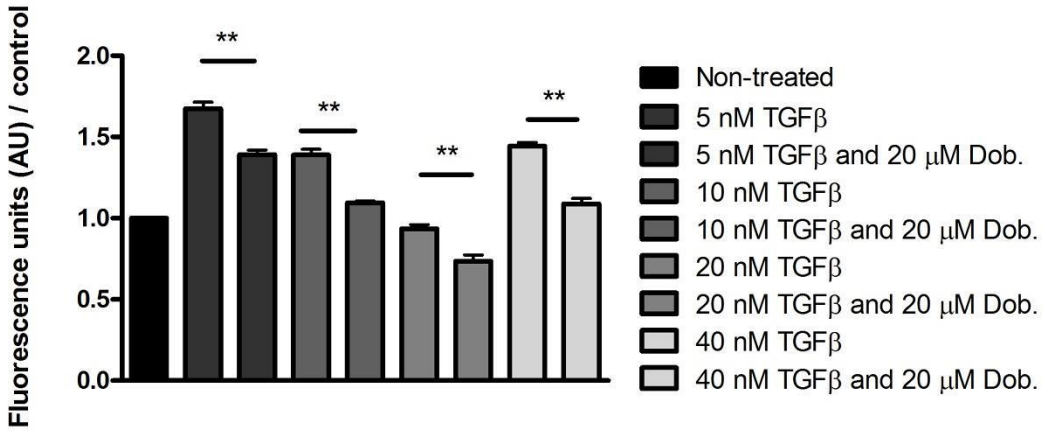

Figure 5.8 TGF-β in combination with dobutamine, reduces paracellular permeability in comparison with TGF-β on its own. Paracellular permeability was assessed after HPAFII have been exposed to TGF-β for 48 h. Values are expressed as mean ± SEM from 3 independent experiments, **p<0.01.

5.3.7 Dobutamine as a possible adjuvant therapy for the treatment of pancreatic cancer

Previous *in vitro* and *in vivo* studies have demonstrated that some β_2–adrenoreceptor agonists inhibited the growth of human breast cancer cells by blocking the Raf-1/ERK 1/2

pathway (Carie and Sebti, 2007). Moreover activation of GPCRs was suggested to be a potential valuable chemotherapeutic strategy in the treatment of a variety of tumours. As an example of this, histamine has been approved to be used in combination with interleukin-2 in the treatment of acute myeloid leukaemia (Toll et al., 2011). In light of previous studies discussed in this thesis, since YAP and Ocln seem to interact as regulators of apical-basal polarity and cell proliferation, it was hypothesised that dobutamine may represent a novel adjuvant treatment for pancreatic cancer by enhancing the cytotoxic actions of the current standard anti-cancer drug gemcitabine. To examine this further, the effect on proliferation rates of AsPc-1 and HPAFII cells following exposure to 20 μM dobutamine in combination with different doses of gemcitabine (AsPc-1 $EC_{50\ Gemcitabine}$: 40 μM; HPAFII $EC_{50\ Gemcitabine}$: 11 μM; Data not shown) (Figure 5.9) was examined. As seen in Figure 5.9-A, proliferation levels were significantly reduced when HPAFII cells were treated with dobutamine in combination with gemcitabine; in AsPc-1, although there was a trend in the reduction of cell proliferation rates after the double treatment, this difference failed to reach statistical significance (Figure 5.9-B). Finally, it was examined if the action of gemcitabine could be potentiated in combination with different doses of dobutamine. When treated with 2 and 5 μM of dobutamine in combination with 40 μM gemcitabine, cell proliferation rate of HPAFII cells decreased in a statistically significant manner (Figure 5.10-A). In AsPc-1, despite the fact that there was a trend in the reduction of cell proliferation, these differences were not statistically significant (Figure 5.10-B). Overall, these results suggest that there seems to be a synergistic interaction between dobutamine and gemcitabine in the context that both compounds given in combination seem to have a stronger anti-proliferative effect *in vitro* than if given alone.

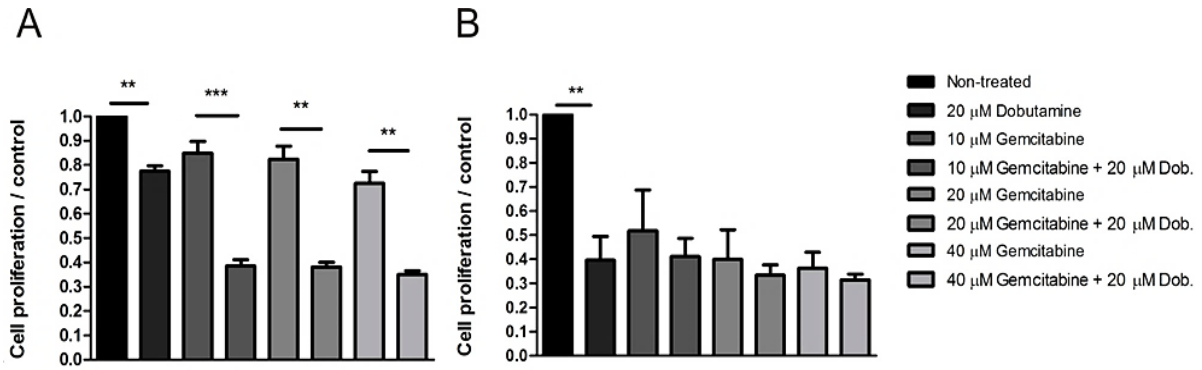

Figure 5.9 Dobutamine enhances the cytotoxic effect of gemcitabine. Cell proliferation was assessed in HPAFII **(A)** and in AsPc-1 **(B)** after cells were exposed to gemcitabine alone or in combination with dobutamine compared to non-treated cells; Values are expressed as mean ± SEM from 3 independent experiments, **p<0.01; ***p<0.001.

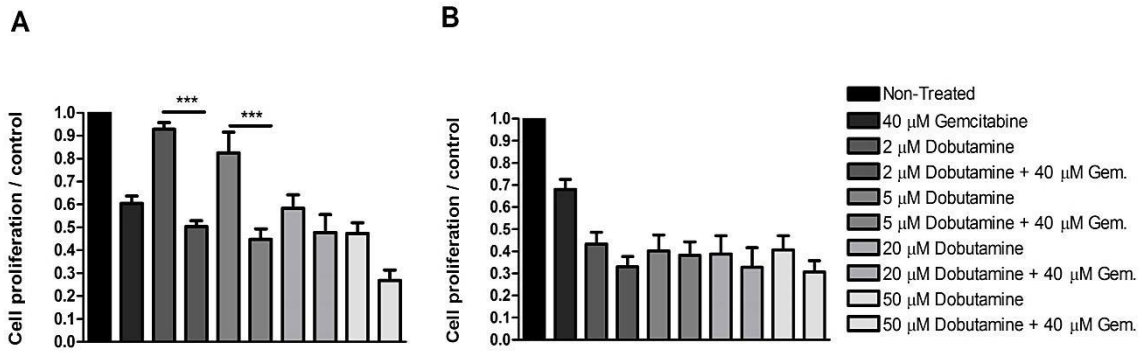

Figure 5.10 Gemcitabine enhances the anti-proliferative effect of dobutamine in HPAFII cells. Cell proliferation was assessed in HPAFII **(A)** and in AsPc-1 **(B)** after cells were exposed to dobutamine alone or in combination with gemcitabine compared to non-treated cells. Values are expressed as mean ± SEM from 3 independent experiments, ***p<0.001.

5.3.8 Dobutamine induces apoptosis in pancreatic cancer cells

The presence of stable TJs has been correlated with the suppression of proliferative pathways and the activation of cell death pathways (Hatakeyama, 2008). Recently there has been a great focus on developing drugs that selectively target apoptotic pathways anticipating a maximization of the therapeutic response while minimizing their toxicity profile. GPCRs have been shown to have a role in activating intra-cellular signal transduction

pathways and, ultimately, cellular responses including programmed cell death (Gu et al., 2000).

This work has already demonstrated that treatment of pancreatic cancer cells with dobutamine, decreased nuclear expression of YAP (Chapter 4, Figure 4.9), sustained cell surface Ocln distribution and re-established cell-cell contacts which in turn suppressed cell proliferation. In the light of previous results, it was hypothesised that dobutamine as a β-agonist might be able to inhibit pancreatic cancer cell proliferation by inducing cell apoptosis. To test this hypothesis, Ocln expression levels were evaluated in response to dobutamine either alone or in combination with gemcitabine (Figure 5.11). As expected, Ocln was detected in whole cell lysates of non-treated HPAFII cells whereas in AsPc-1 cells Ocln expression was diminished. When treated with a single dose of dobutamine, Ocln levels remained approximately the same in non-treated HPAFII cells while on AsPc-1 this increase was more than two-fold higher. Gemcitabine treatment had the opposite effect in terms of Ocln expression, in both cell lines. In HPAFII, gemcitabine induced a decrease in Ocln expression. This could partially be explained by its mechanism of action. Being a pro-drug, gemcitabine requires a phosphorylation step by deoxycytidine kinase to an active form that then gets incorporated into DNA, ultimately causing cell death (Plunkett et al., 1995). It might be that the gemcitabine exposure time as well as the concentration used in these studies was not optimal for this specific cell line. In AsPc-1 cells, Ocln levels after dobutamine treatment were higher compared to non-treated levels; when combined with gemcitabine, there was a statistically significant increase in Ocln expression levels in both cell lines, being more pronounced however in the AsPc-1 *in vitro* model (Figure 5.11).

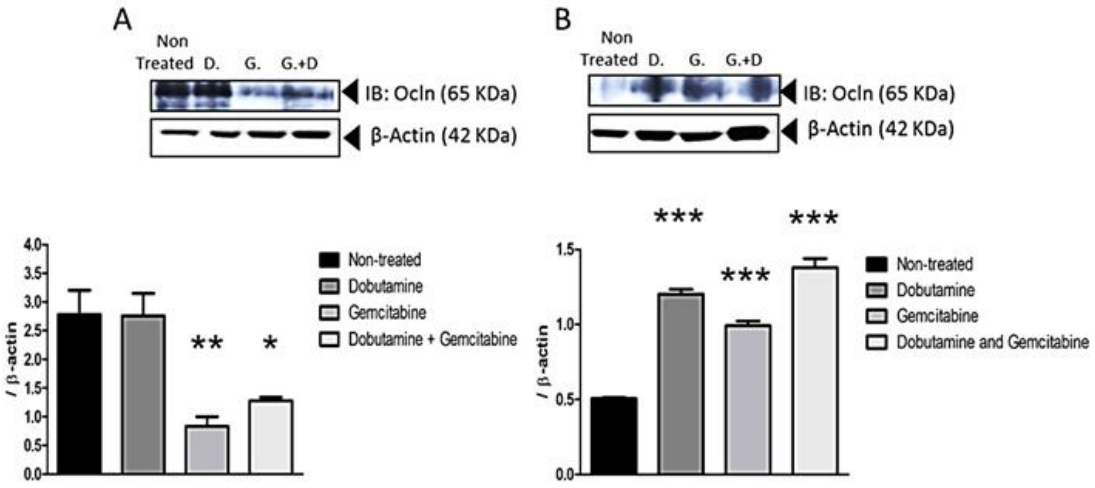

Figure 5.11 Dobutamine and gemcitabine combined treatment increased Ocln production in AsPc-1 cell lines. (A) Immunoblot showing Ocln levels after HPAFII cells were treated with 20 μM dobutamine either alone or in combination with 40 μM gemcitabine during a 48 h period; **(B)** Immunoblot showing Ocln levels after AsPc-1 cells were treated with 20 μM dobutamine either alone or in combination with 40 μM gemcitabine during a 48 h period. Values are expressed as mean ± SEM from 3 independent experiments, p*<0.05; **p<0.01; ***p<0.001 compared to non-treated levels; ***Abbreviations: D. – Dobutamine; G. – Gemcitabine; G.+ D. – Gemcitabine and dobutamine***

To check for apoptosis induction, different assays were carried out after cells were treated with dobutamine and gemcitabine, either alone or in combination. As previously discussed in section 2.12, Chapter 2, any cell that has been committed to apoptosis will have exposed phosphatidylserine residues in the outer cell membrane that will bind to annexin V (Krysko et al., 2008); however in the late stages of apoptosis, if the cell membrane is compromised, any residue inside the cell will stain positively as well. Only a negligible fraction of DNA staining could be observed in AsPc-1 cells treated with dobutamine only, whereas in both cell lines treated with gemcitabine, apoptotic and necrotic features were equally observed. PI is a marker of late stage apoptosis, as its ability to enter a cell is dependent upon the permeability of the membrane, thus PI does not stain live or early apoptotic cells due to the presence of an intact plasma membrane (Vermes et al., 2000). This data suggests that dobutamine and gemcitabine treatment elicit apoptotic events and at

this specific time course of 48 h, the combined treatment has also triggered a necrotic response in HPAFII cells (Figure 5.12).

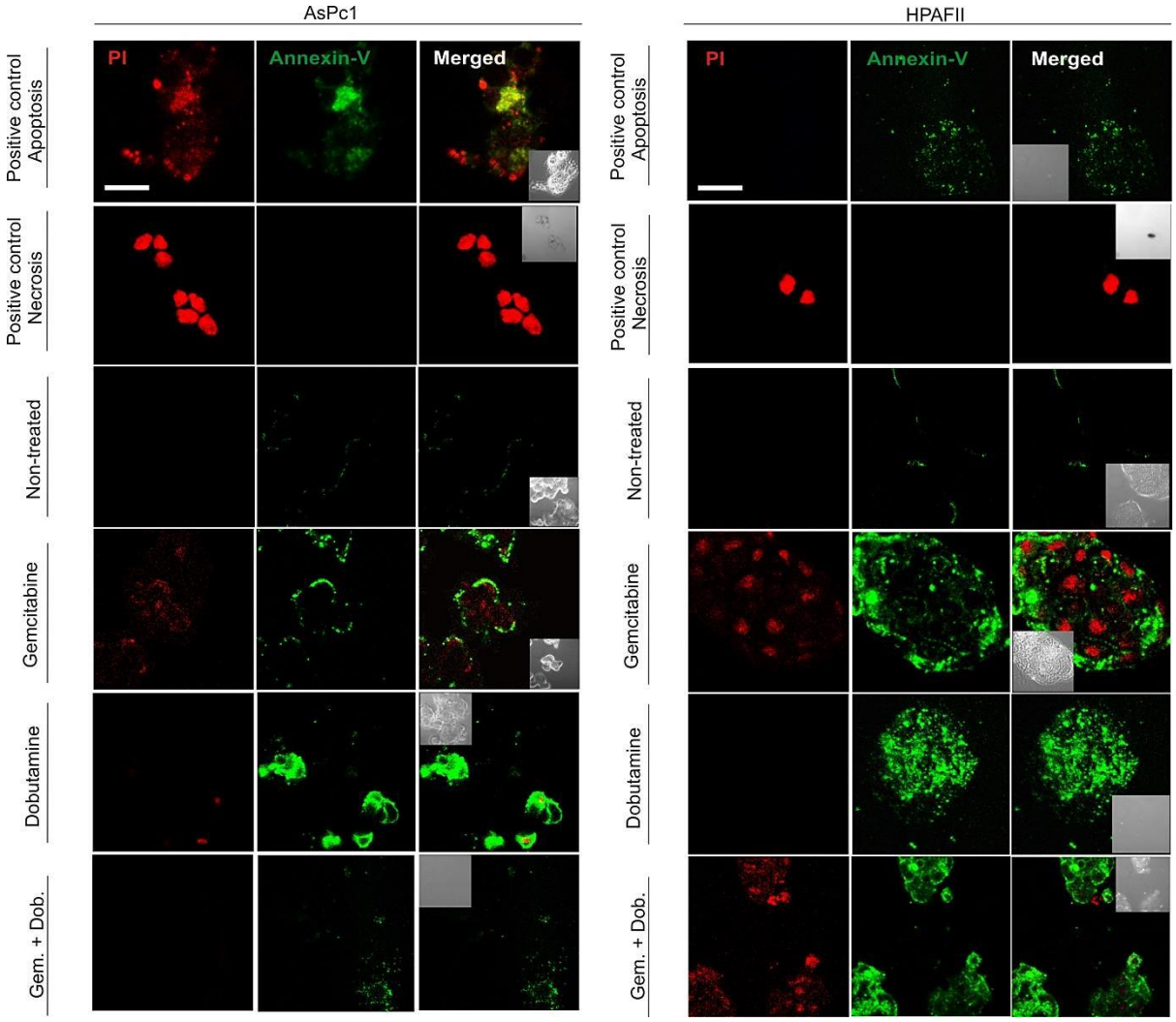

Figure 5.12 Dobutamine induces apoptosis in the pancreatic cancer cell line models: AsPc-1 and HPAFII. Annexin V/PI staining in AsPc-1 (left hand side) and HPAFII cells (right hand side) after being exposed to 40 μM gemcitabine and 20 μM dobutamine either in combination or alone for a 48 h period; scale bar, 20 μm. ***Abbreviations: Gem- Gemcitabine; Dob- Dobutamine*.**

Apoptosis and necrosis refer to two mechanistically different processes in the context that necrosis is the passive result of cellular injury whereas apoptosis forms an integral part of physiologically normal processes (Vermes et al., 2000). Apoptosis ensures a dynamic equilibrium between cell proliferation and cell death, playing a crucial role in the size of cells

and tissues. In malignancy, however, resistance to apoptosis has been shown to be one of the hallmarks of cancer, as outlined by Hanahan and Weinberg (Chapter 1: Figure 1.11, (Hanahan and Weinberg, 2000). It was predicted that apoptosis would be the primary mechanism of cell death induced by dobutamine and/or gemcitabine. All the experiments performed were based on a 48 h time window; so that, the percentage of apoptotic versus necrotic stained cells was examined after cells were treated with dobutamine, either alone or in combination with gemcitabine during a 48 h time-course. Dobutamine treatment had a slightly more pronounced effect in prompting an apoptotic cell response in HPAFII cell line model: ~38.2 % compared to AsPc-1 cells: ~22.7 % (Figure 5.13). No PI/necrotic staining was detected in HPAFII and only negligible amounts could be observed in AsPc-1 cells (~5.8%) consistent with the data showed in Figure 5.12. Treatment with gemcitabine alone had a stronger effect in HPAFII (~57.3 %) than in AsPc-1 cells (~24.9 %), which was expected given the different aggressiveness profile of these two cell lines (Figure 5.13). It was anticipated that dobutamine treatment would enhance the cytotoxic actions of the current standard chemotherapeutic drug, gemcitabine. Unexpectedly, however, the combined treatment of dobutamine and gemcitabine had a very subtle effect on stimulating an apoptotic and/or necrotic response. In both cell lines, ~50 % of cells were alive, and only ~11.5 % and 24.4 % of HPAFII and AsPc-1 cells respectively, were labelled as apoptotic and necrotic (Figure 5.13). These observations were not consistent with the annexin/PI immune-labelling as shown in Figure 5.12 as the combined treatment produced a marked effect in AsPc-1 cells as no adherent cells could be observed, and in HPAFII cells, both apoptotic and necrotic staining could be detected (Figure 5.12).

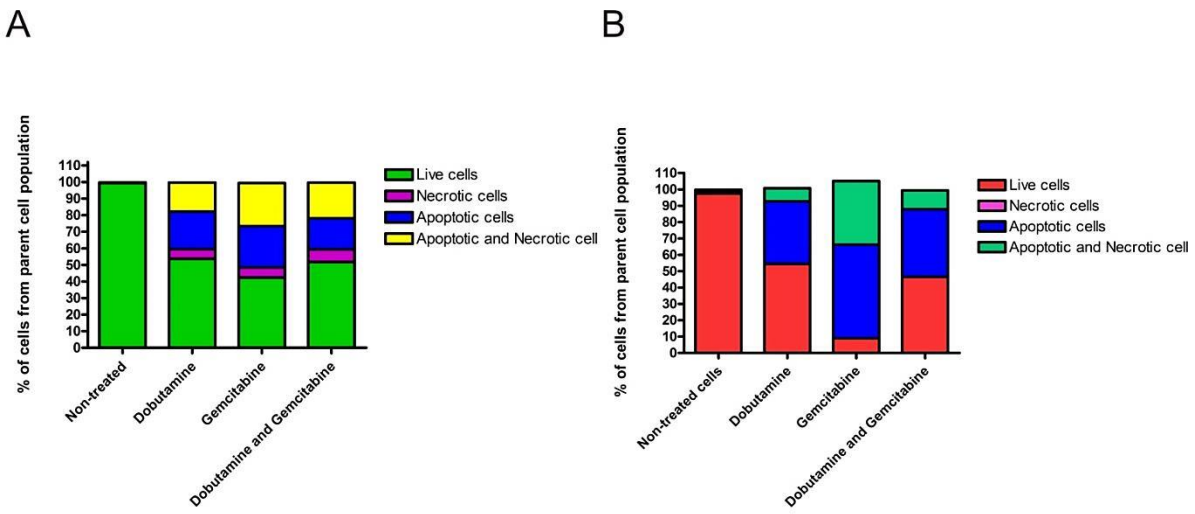

Figure 5.13 Fast growing and aggressive cells, AsPc-1, were more resistant to the effects of gemcitabine either alone or combined with dobutamine compared to HPAFII. Histogram showing the percentage of apoptotic and/or necrotic AsPc-1 **(A)** and HPAFII **(B)** cells from parent cell population after annexin/PI staining through FACS analysis.

The features of end stage apoptosis overlap with necrotic parameters such as compromised membrane integrity and leakage of proteins in the supernatant (Denecker et al., 2001). To circumvent this, an enzyme-linked immunoabsorbent assay (ELISA) was used to quantify DNA fragmentation after cells were exposed to gemcitabine and dobutamine either alone or in combination. To evaluate the presence of histones and nucleosomes in cell lysates of AsPc-1 and HPAFII cells after being exposed to different concentrations of dobutamine and/or gemcitabine, absorbance measurements were taken at 490 nm (Figure 5.14 and Figure 5.15). Both cell lines showed a similar behaviour in terms of apoptotic induction after exposure to either combined or single treatment. AsPc-1 cells treated with a fixed dose of dobutamine supplemented with an increasingly higher concentration of gemcitabine showed concentration-dependent cell death response (Figure 5.14), consistent with the proliferative response (Figure 5.9). Conversely, HPAFII cells treated with a fixed dose of gemcitabine combined with increasingly higher concentrations of dobutamine showed considerably higher nucleosome detection compared to cells treated with a fixed dose of dobutamine and

increasingly higher concentrations of gemcitabine, consistent with the proliferative response observed in Figure 5.10.

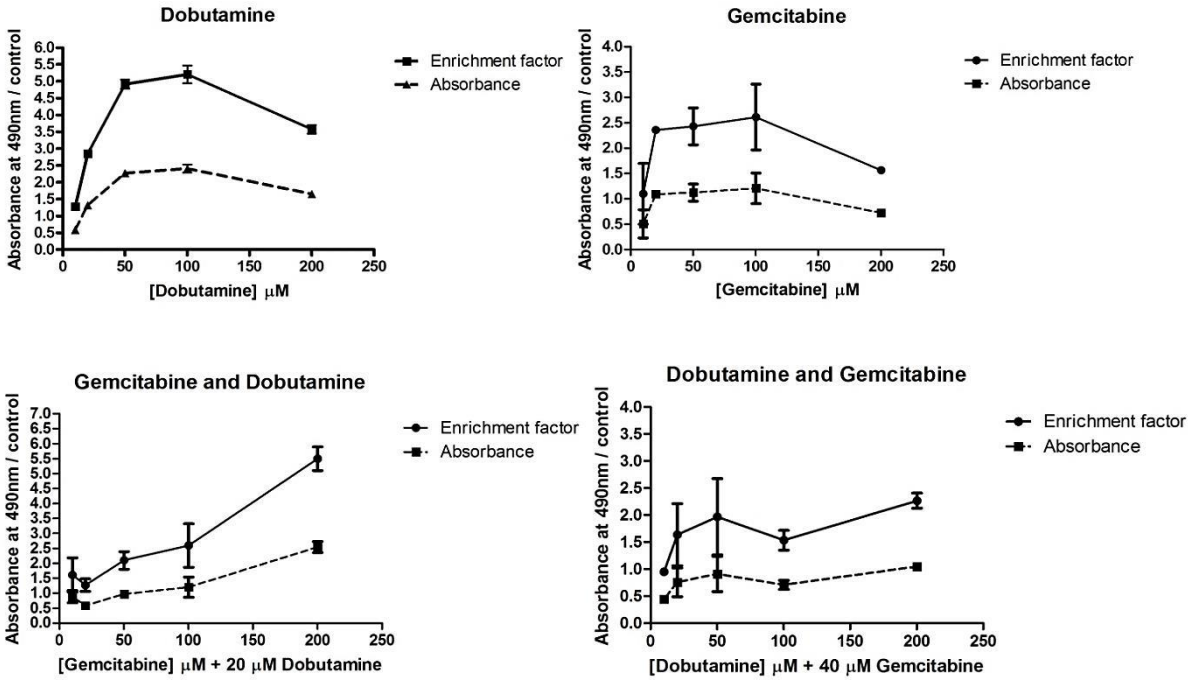

Figure 5.14 Gemcitabine and dobutamine treatment of AsPc-1 cells was more efficient at inducing an apoptotic response than a single treatment. AsPc-1 cells were cultured at different concentrations with dobutamine and gemcitabine either alone or in combination, for a 48 h period. After cell lysis, the cytoplasmic fractions were pre-diluted 1:10 with incubation buffer and then analysed through an ELISA immunoassay and absorbance measurements were taken at 490 nm. Enrichment factor was calculated by dividing the absorbance of dying/dead cells by the corresponding control (viable cells). Each data point refers to the absorbance of dead cells divided by the absorbance obtained from the non-treated sample (without a cell-death inducing agent). Data presented are the mean ± SEM of triplicates from three independently performed experiments.

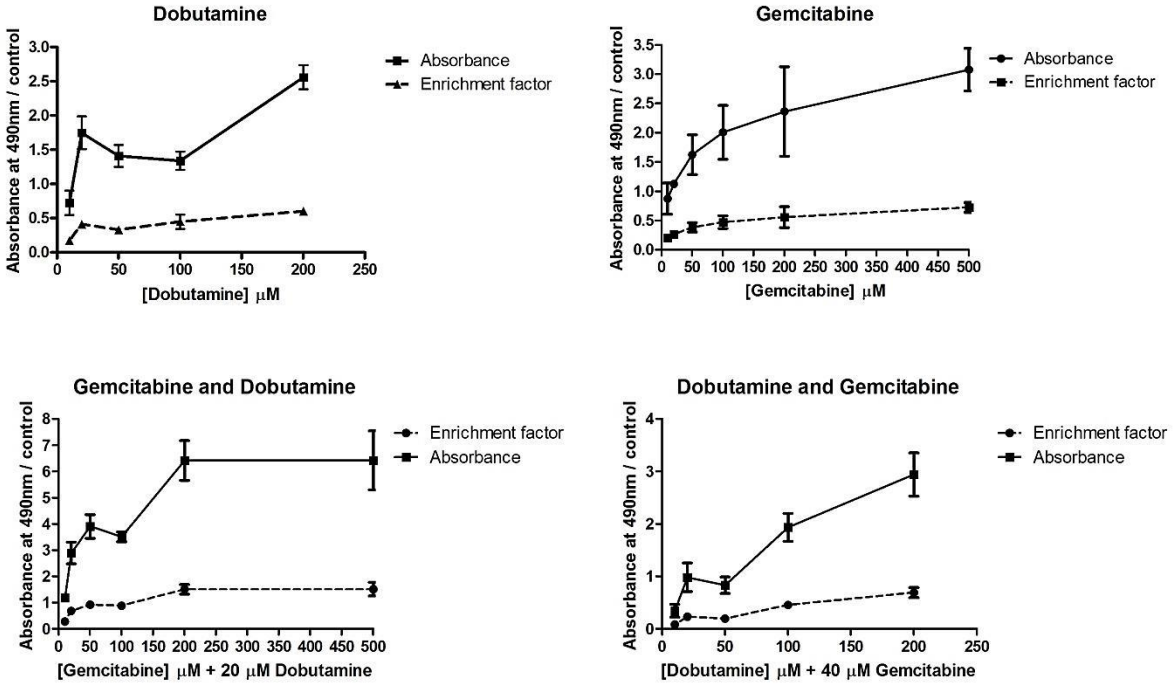

Figure 5.15 Combined treatment of gemcitabine and dobutamine in HPAFII cells was more efficient than single treatments in inducing an apoptotic cell response. HPAFII cells were cultured at different concentrations with dobutamine and gemcitabine either alone or in combination, for a 48 h period. After cell lysis, the cytoplasmic fractions were incubated with buffer and then analysed through an ELISA immunoassay; absorbance measurements were taken at 490 nm. Enrichment factor was calculated by dividing the absorbance of dying/dead cells by the corresponding control (viable cells). Each data point refers to the absorbance of dead cells divided by the absorbance obtained from the non-treated sample (without a cell-death inducing agent). Data presented are the mean ± SEM of triplicates from three independently performed experiments.

5.3.9 Compound C and dobutamine as a possible adjuvant therapy in the treatment of pancreatic cancer

Despite the aggressive surgical treatment combined with adjuvant chemotherapy and radiotherapy, pancreatic cancer remains one of the deadliest epithelial cancers. Lack of selectivity and specificity are still the main hurdles that contribute to such unsuccessful outcome. To overcome this, a potential new therapeutic avenue was explored by directing the uptake of a specific gemcitabine chimera that can act as a substrate of PepT1. Studies

have also suggested the involvement of α_2–adrenergic receptors in the transport of peptidomimetic drugs in cultured epithelial cells devoid of innervation (Berlioz et al., 2000). Adrenergic receptors have been shown to stimulate intestinal absorption of the β-lactam antibiotic cephalexin through oligopeptide transport in a human intestinal cell line. (Berlioz et al., 2000). Clonidine, an α_2–agonist, has been reported to cause the stimulation of cephalexin uptake through PepT1; this stimulatory effect was hypothesised to be the consequence of a higher affinity of the oligopeptide transporter for its substrate and/or an increase in the number of functionally available transporter molecules located at the apical side of Caco 2 (Berlioz et al., 2000).

As it was previously shown, PepT1 provides a potential strategy to target pancreatic cancer cells for the delivery of chimeric agents designed to affect cell proliferation and viability. We have tested this concept using chemically modified gemcitabine chimeras. After showing the suppression of cell proliferation induced by a β-agonist, we sought to test the potential cytotoxic action resulting from the combined treatment of dobutamine and the PepT1 specific gemcitabine containing compound C.

AsPc-1 PepT1$^{-/-}$ cell line and AsPc-1 WT were exposed to compound C either alone or in combination with dobutamine during a 48 h period (Figure 5.16). Cell proliferation rate was reduced after PepT1 expressing cells were treated with the combined treatment in comparison to the single treatment (Figure 5.16-B), even though statistical significance was only obtained for 5 μM of gemcitabine. As expected, dobutamine given in combination with the compound C had no effect on PepT1$^{-/-}$ cells. To further support this evidence, a study carried out by Sundaram and co-authors described that after stimulation of α_2–adrenergic receptors by clonidine, the Na^+/H^+ exchanger activity increased due to a higher intracellular pH in enterocytes (Sundaram, 1995). The decrease in cell proliferation observed in the

AsPc-1 cell line could be partially explained by an increased affinity induced by the β-agonist, dobutamine, towards the PepT1 specific gemcitabine chimera.

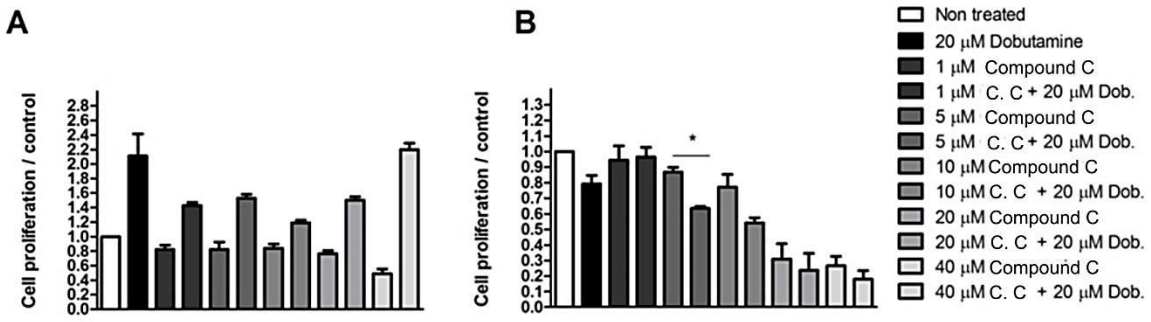

Figure 5.16 Dobutamine did not affect PepT1$^{-/-}$ cells. Cell proliferation assay after AsPc-1 PepT1$^{-/-}$ **(A)** and AsPc-1 **(B)** cells were treated with Compound C alone or in combination with 20 μM dobutamine during a 48 h period. ***Abbreviations: C. C – Compound C***

Quite striking and unexpected was the lack of effect of dobutamine treatment on PepT1$^{-/-}$ cell line; proliferation rates were two-fold higher compared to non-treated cells. This prompted us to further characterize cell proliferation and viability of the PepT1$^{-/-}$ cell response towards dobutamine treatment. In terms of cell proliferation, PepT1$^{-/-}$ cells presented an $EC_{50\ Dobutamine}$ nearly three-fold higher (98.4 μM) than the WT AsPc-1 (39.8 μM) (Figure 5.17-top panel and Figure 5.2). With regards to cell viability, the $EC_{50\ Dobutamine}$ obtained for PepT1$^{-/-}$ was nearly two-fold higher (207 μM) when compared to the WT cell line (117 μM) (Figure 5.17-bottom panel and Figure 5.2).

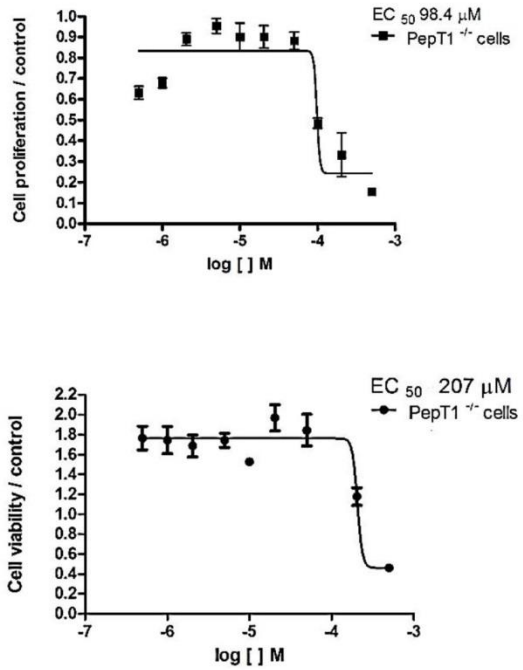

Figure 5.17 Cell proliferation and viability studies after PepT1$^{-/-}$ cells were treated with dobutamine for a 48 h period. Sigmoidal dose response curves showing the EC_{50} values obtained for cell proliferation and viability response during a 48 h time course. Values are expressed as mean ± SEM from 3 independent experiments.

5.4 Summary of results

- The suppression of cell proliferation induced by dobutamine in AsPc-1 cells is β-adrenergic mediated;
- Dobutamine suppresses cell proliferation *in vitro* in pancreatic cancer cells: AsPc-1 and HPAFII;
- Dobutamine inhibition of HPAFII and AsPc-1 cell proliferation involves Ocln;
- Dobutamine treatment reduces size-selectivity of TJ paracellular permeability;
- Dobutamine rescues the effect of the ROCK inhibitor, Y-27632, through a mechanism involving Ocln reactivation;
- Dobutamine rescues the effect of cell polarity disruption induced by TGF-β;
- Dobutamine is a possible adjuvant therapy for the treatment of pancreatic cancer;
- Dobutamine induces apoptosis in pancreatic cancer cells: AsPc-1 and HPAFII;
- PepT1$^{-/-}$ cells are less responsive to dobutamine actions resulting in different cell proliferation and viability responses.

5.5 Discussion

In this chapter, dobutamine was presented as a potential adjuvant drug molecule with a direct application in the treatment of pancreatic cancer. Previously showed to induce YAP translocation from the nucleus to the cytoplasm (Figure 4.9, Chapter 4), dobutamine was evaluated as a possible anti-proliferative molecule (Figure 5.1). Consistent with Diep's observations showing that down-regulation of YAP expression reduces cell proliferation and clonogenicity of pancreatic cancer cells (Diep et al., 2012), we have confirmed that through YAP translocation, dobutamine is also able to suppress cell proliferation in AsPc-1 and

HPAFII cells (Figure 5.1). Comparing pancreatic cell line viability and proliferation responses to dobutamine treatment, the compound appeared to produce a more pronounced effect in decreasing cell viability in HPAFII than in AsPc-1 cells and decreased cell proliferation in a slightly more pronounced manner in AsPc-1 compared to HPAFII cells (Figure 5.2).

Fujita and co-authors suggested that β-adrenergic stimulation supresses microglia proliferation (Fujita et al., 1998) and Junker reported that such stimulation confers neuroprotection (Junker et al., 2002). More recently, adrenaline and norepinephrine, have been reported to inhibit cell proliferation in rat gastric mucosa (Olaleye et al., 2010). It was now shown that another catecholamine, dobutamine, was able to reduce cell proliferation in two *in vitro* models of pancreatic cancer in a time and dose-dependent manner (Figure 5.1).

To check if the suppression of cell proliferation induced by dobutamine was β-agonist mediated, cells were treated with the selective β_1 receptor antagonist, atenolol and with the non-selective antagonist propranolol (Figure 5.3, Chapter 8: Figure 8.6). Our results confirmed that the suppression of proliferation in several pancreatic cancer cell lines induced by dobutamine was β-adrenergic mediated. The β_1 adrenergic receptor is a GPCR associated with the Gs heterotrimeric G-protein and is expressed extensively in cardiac tissue (Ungerer et al., 1993). In pancreas, GPCRs are a major class of receptors and have been reported to be involved in islet cell signalling mediating the extracellular messages to intracellular signalling pathways (Winzell and Ahren, 2007). In exocrine pancreas, PDAC has been reported to frequently overexpress the enzymes Cox-2 (Yip-Schneider et al., 2000) and 5-lipoxygenase (Hong et al., 1999) that mediate the metabolic conversion of arachidonic acid into prostaglandins, thromboxanes and leukotrienes (Goetzl et al., 1995). Since the physiological effects of prostaglandins are mediated in part by GPCRs (Breyer et al., 2001), we assessed the level of for β_1 – adrenergic receptor expression in the

pancreatic cancer cell lines that had been examined in these studies. According to a public dataset, (http://www.oncomine.org) the ductal adenocarcinoma derived cell lines, AsPc-1 and HPAFII, both express β_2 adrenergic receptors but only AsPc-1 expresses the β_1 sub-type. This data suggests that the observed effects of dobutamine, which acts predominantly on β_1 receptors, on cell proliferation are mediated by β_1- adrenoreceptors; this was observed by the reduction in cell proliferation induced by the selective antagonist atenolol when given alone (Chapter 8, Figure 8.6) and relatively unaffected cell proliferation levels when the antagonist selectively counteracted the agonist effects (Figure 5.3).

In this study, β-adrenergic stimulation through a single cell proliferation assessment was assessed. To fully address β-adrenergic involvement in pancreatic cancer cells in response to dobutamine, cAMP levels should have been measured. Elevation of intracellular concentration of cAMP is a direct result from agonist binding (Pak et al., 2002). An understanding of the specific role of β-receptor expression and function in pancreatic cancer will involve a more robust knowledge about receptor concentration in a specific type of cell line as well as the persistence of receptor activity after being induced by an agonist. In order to confirm that the reduction in proliferation and viability was mediated through β-adrenergic receptors, mRNA expression for β_1- and β_2- adrenergic receptors in both cell lines needs to be assessed. Additionally, an analysis of the transcription factors and genes targeted by β-adrenergic stimulation and their role in the dobutamine anti-proliferative effect should be carefully explored. Further studies will need to address how downstream factors such as protein kinase and phosphorylated proteins respond to β-adrenoreceptor activation in the context of cell proliferation inhibition.

Nevertheless, this work has shown some therapeutic potential in targeting β-adrenoreceptors in cancer. Being expressed at the cell surface, the hurdles posed by drug

penetration to reach their target can be overcome and thus would not be subject to loss of efficacy from the action of multidrug resistance transporters. However, a downside worth mentioning involving the targeting of β-adrenoreceptors in cancer concerns the fact that, given their relatively narrow pharmaceutical window, any therapeutic strategy based on receptor targeting should have only short-term side effects. Major problems in adrenergic therapy involve the fact that receptor down-regulation limits the potential in using β-agonists in controlling cell replication. Conversely prolonged receptor stimulation generates a loss of functional response leading to the sequestration of receptor proteins (Slotkin et al., 2000).

Carcinogenesis is perceived to be a multistep process that arises from a disrupted balance between cell proliferation and apoptosis. Molecular pathways involved in tissue homeostasis, such as the Hpo pathway, are critical in establishing a tight control between cell division and death. The Hpo pathway poses therefore as a prime target for dysregulation in cancer. A hallmark of aggressive epithelial cancers, such as PDAC, is the loss of cell-cell contacts and resistance to apoptosis induction with these contacts, in particular those involving Ocln, being related to suppressing cancer progression and metastasis (Martin et al., 2010; Tobioka et al., 2004a).

A number of TJ-associated proteins have been demonstrated to regulate epithelial cell proliferation, with some of these proteins being directly related to pathways and mechanisms that have been linked to cytoplasmic elements and to transcriptional factors. In the previous chapter, modulation of YAP function was shown to facilitate Ocln stabilization at the plasma membrane as well as increasing the extent of functional TJs. Now it was shown that knock-down of Ocln levels in dobutamine treated cells resulted in no proliferation changes in the fast growing cell line AsPc-1 compared to non-treated cells. In HPAFII, however, knock-down of Ocln produced a non-significant decrease in proliferation rate compared to

the control, but significantly higher compared to the wild type (Figure 5.4). These observations highlight the potential role of Ocln as an extracellular sensor that could coordinate the completion of apical-basal polarity with the suppression of lateral cell proliferation.

Although the Ocln knockout mouse suggests that Ocln does not play an essential role in epithelial barrier function *in vivo* (Saitou et al., 2000), mutant and chimeric Ocln expression has been shown to affect the regulation of paracellular permeability (Balda et al., 2000). Bamforth and co-workers also reported that in murine submandibular gland carcinoma cells, the expression of an N-terminally truncated mutant of Ocln caused a decrease in TER simultaneously with an increase in paracellular permeability (Bamforth et al., 1999). Retention of Ocln at the plasma membrane and its ability to interact with YAP and TEAD would shift the distribution of these co-activation factors from the nucleus to reduce their proliferative actions and increase the Ocln-mediated TJ barrier properties (Al-Sadi et al., 2011). To test this, the rate of 4 kDa fluorescent dextran as a marker for paracellular flux was evaluated (Matter and Balda, 2003). Paracellular permeability in HPAFII monolayers decreased significantly after dobutamine was applied at the apical side of these cells (Figure 5.5). This confirmed that the dobutamine induced effects on Ocln stabilization at the cell membrane contributed to the TJ integrity in this system.

Loss of Ocln expression or disruption of its normal cellular organization is associated with epithelial cell transformation (Li and Mrsny, 2000). PKCζ participates in both polarity complex function and Hpo signalling pathways and Ocln has been shown to selectively bind to this molecule through a C-terminal coiled-coil domain (Nusrat et al., 2000). To test the involvement of PKCζ in processes involving stabilization of Ocln at the cell membrane through dobutamine mediation, HPAFII monolayers were incubated with PKCζ inhibitor. As

expected and given its role in cell polarity, PKCζ-PS caused a drop in TER values and treatment with dobutamine failed to correct this outcome (Figure 5.6). These observations are consistent with previous roles of PKCζ in being critical for TJ structure/function through its actions with Ocln (Nusrat et al., 2000). ROCK phosphorylates the regulatory subunit of MLC (myosin light chain kinase) and it also inhibits the action of phosphatases which in turn inhibits MLC phosphorylation (Totsukawa et al., 2000). Not surprisingly, TER values dropped after exposing cells to the ROCK inhibitor, which could possibly be due to the sustained activation of phosphatases and consequently the inhibition of MLC phosphorylation, essential for the actin-myosin interaction in the cytoskeleton. Because TJs play a crucial role in regulating paracellular permeability and this seems to be influenced by ROCK inhibition, we analysed mechanisms by which modulation of Ocln re-expression through dobutamine exposure could regulate epithelial barrier integrity. In this study, enhanced permeability induced by ROCK inhibition could be partially rescued by dobutamine and this mechanism appeared to be Ocln mediated (Figure 5.7). These studies support the hypothesis that ROCK may influence not only the maintenance of normal actin dynamics (Ishizaki, 2000) but might also affect epithelial TJ function.

TGF-β has been found to be abundantly expressed in malignant epithelial tissues (Derynck et al., 1985); studies have suggested that these particular molecules were able to induce the disruption of TJs and the acquisition of a mesenchymal phenotype in breast epithelial culture models (Seton-Rogers et al., 2004). Besides, Hpo signalling is involved in cross talk with other signalling pathways including TGF-β/SMAD (Varelas et al., 2010) and an association between Ocln and TGF-β signalling has been reported in murine hepatocytes (Barrios-Rodiles et al., 2005; Ozdamar et al., 2005). The second extracellular loop of Ocln, which is essential for reversing Raf-driven epithelial to mesenchymal events (Li and Mrsny, 2000), functions as a binding site to bring TGF-β receptor I to TJ structures where it can

control cell density through SMAD-based transcriptional events (Varelas et al., 2010). As dobutamine is involved in Ocln stabilization at the cell membrane, we sought to test if it would affect the biological role of TGF-β in TJ integrity by measuring paracellular permeability. As it has been previously reported, TGF-β displays a bell-shaped dose-response (Lebrin et al., 2005). In this system, at low concentrations (5, 10 and 20 nM), the cytokine stimulated TJ formation and consequently permeability reduction, whilst at higher concentrations (40 nM) it did not (Figure 5.8). TGF-β disrupted functional TJs and increased cellular permeability. Consequently dobutamine was able to significantly recover the paracellular permeability loss induced by TGF-β treatment (Figure 5.8). This is consistent with the partial recovery in TER values after dobutamine application in combination with the ROCK inhibitor (Figure 5.7). Being involved in mediating the growth inhibitory effects of TGF-β, dobutamine modulation of Rho/ROCK pathway represents an attractive approach to target pancreatic cancer. To fully address the effect of TGF-β on HPAFII cells, a fresher and lower passage number of these cells should have been cultured and TER values should have been assessed in parallel with the paracellular permeability studies. Also, another valuable approach would be to follow dobutamine actions after cells were treated with TGF-β inhibitor negatively affecting SMAD2 phosphorylation.

Given the fact that YAP is hyper-activated in many human malignancies (Baldwin et al., 2005; Overholtzer et al., 2006; Snijders et al., 2005; Steinhardt et al., 2008; Zender et al., 2006) together with its role in promoting cell proliferation (Chen et al., 2010) and inhibiting cell death (Strano et al., 2005), any therapeutic intervention for cancer would involve reducing or ablating the oncogenic function of YAP. Studies have suggested that by reducing YAP activity, tumour growth suppression would be effective (Diep et al., 2012; Lamar et al., 2012; Wang et al., 2012; Zhang et al., 2012). Huang and co-workers, reported that knock-down of YAP would increase the sensitivity of cancer cells to standard

chemotherapeutic agents such as cisplatin, erlotinib and the small molecule antagonist of surviving, S12 (Huang et al., 2013). Dobutamine has been reported to inhibit YAP nuclear accumulation and YAP-mediated transcriptional activation (Bao et al., 2011). This compound was examined as a possible adjuvant molecule in the current standard treatment of pancreatic cancer. HPAFII cells treated with dobutamine showed a synergistic decrease in cell proliferation when treatment was combined with gemcitabine but AsPc-1 did not (Figure 5.9 and Figure 5.10). This was probably due to an insufficient exposure time to the combined treatment in this particular cell line. These findings suggest that dobutamine might increase sensitivity towards gemcitabine treatment in pancreatic cancer cells. The use of other cancer drugs, in this case gemcitabine, in combination with YAP targeted therapies, posed therefore as an attractive strategy that could maximize therapeutic outcome.

Next, it was investigated if not only dobutamine was able to trigger an apoptotic response in pancreatic cancer cells, but it was also explored if a combined treatment of dobutamine and gemcitabine would maximize the apoptotic response followed by necrosis. It was speculated that dobutamine was able to inhibit proliferation and induce apoptosis in pancreatic cancer cells through the stabilization of Ocln at the cell membrane. Studies have reported the function of Ocln in a fail-safe mechanism for cell death induction (Liu et al., 2012; Osanai et al., 2006; Rachow et al., 2013). Further to this, Ocln expression in Raf-activated epithelial cells activates multiple apoptotic pathways such as Bid, Caspase 6 and 12 (Mrsny, et al., in preparation). Dobutamine treatment significantly induced Ocln production in the fast and aggressively growing AsPc-1 cells, whereas in HPAFII, Ocln levels remained approximately the same as in non-treated levels (Figure 5.11).

Apoptosis is a multi-step cellular event that starts with cell shrinkage; initially the cell membrane remains intact until caspases are activated leading to intracellular shifts in Ca^{2+}

and pH that result in the mitochondrial membrane potential (MMP^2) function impairment and lysosomal membrane pumps lose their function. At this stage, endogenous endonucleases are activated and phosphatidylserine gets redistributed culminating in cell disintegration into apoptotic bodies (Vermes et al., 2000). It is very challenging, not only from the conceptual point of view but also from a technical perspective, to detect apoptosis in a cell system. There is no inflammatory reaction involved and as well as this the apoptotic bodies due to their reduced size undergo rapid phagocytosis, and scattered single cells might escape detection at the early stages of apoptosis (Wyllie et al., 1980). Through a simple annexin and PI staining, it was confirmed that dobutamine as a single treatment induced an apoptotic response in both AsPc-1 and HPAFII and when combined with gemcitabine elicited apoptotic events followed by a necrotic response in HPAFII cells (Figure 5.12). Next, cells stained for annexin/PI through FACS, were analysed. Unpredictably, dobutamine and gemcitabine had a very subtle effect on stimulating an apoptotic and/or necrotic response (Figure 5.13). It is possible to postulate that due to receptor down-regulation, the apoptotic induction stimulated by β-agonists may not be sufficient to trigger a more robust apoptotic response. However, the 48 h time point and the doses tested were the chosen to be consistent with the previous data showing the activity modulation of specific Hpo pathway elements in response to dobutamine. Finally, through a specialised ELISA immunoassay, the presence of nucleosomes was detected in cells treated with a fixed dose of dobutamine complemented with increasingly higher concentrations of gemcitabine or vice versa. Together, this data suggests that dobutamine in combination with gemcitabine induces Ocln production in AsPc-1 cells and an apoptotic response measured through annexin staining and nucleosome detection (Figure 5.14 and Figure 5.15). Dobutamine seems to have the potential to work as an adjuvant to conventional chemotherapeutics for the treatment of pancreatic tumours that have high levels of YAP expression.

As this data suggests, dobutamine might represent a promising adjuvant molecule in the treatment and management of pancreatic cancer. After showing PepT1 over-expression in pancreatic cancer cell lines (Chapter 3), the possibility of increasing therapeutic efficacy using a gemcitabine-amino acid chimera that functions as PepT1 substrate together with dobutamine was explored (Figure 5.16). As anticipated, the cell proliferation rate was reduced after PepT1 expressing cells were treated with the dobutamine and gemcitabine. In PepT1$^{-/-}$, however, this effect was not observed. cAMP plays a crucial role in controlling the movement of hydroelectrolytic particles across the intestinal epithelium (Schimmel et al., 1980). PepT1 transport is proton dependent, so it seems likely that the β-agonist dobutamine might favour the uptake of gemcitabine in the cells. Nevertheless, to determine if administration of dobutamine results in a rapid enhancement of pancreatic absorption of gemcitabine through increased activity of PepT1, a cell line stably expressing alpha or β-adrenergic receptors and PepT1 should be used as a model to determine whether alpha or β-agonists can directly modulate the activity of PepT1 in the absence of nerve endings (Berlioz et al., 2000).

The possibility of other β-adrenoreceptor subtypes being involved in the mechanism described was not investigated. Enhanced absorption of peptides and peptidomimetic drugs opens up new avenues for the therapeutic application of β_1 agonists as potential new drug delivery agents. To pursue such a line of study should incorporate efforts to examine the irresponsiveness of PepT1$^{-/-}$ cells to dobutamine. In order to address this, $EC_{50\ Dobutamine}$ values were assessed in the AsPc-1 WT and PepT1$^{-/-}$ cell lines (Figure 5.17). In terms of cell proliferation, PepT1$^{-/-}$ cells presented a much higher $EC_{50\ Dobutamine}$ compared to the WT (Figure 5.17-top panel and Figure 5.2). In terms of cell viability, the $EC_{50\ Dobutamine}$ obtained for PepT1$^{-/-}$ was higher compared to the WT cell line (Figure 5.17-bottom panel and Figure

5.2). All together these results suggest that dobutamine has a stronger cytotoxic potential in AsPc-1 expressing PepT1 compared to AsPc-1 PepT1$^{-/-}$ cells.

Our experiments using dobutamine as a β_1 -adrenergic agonist, show that it can slow pancreatic cancer cell proliferation by re-establishing cell-to-cell contacts and augmenting gemcitabine-induced apoptosis (Figure 5.18). To establish the potential utility in the use of sympathomimetic drugs as a therapeutic strategy, studies using *in vivo* models will be required. Similarly, neuropeptide therapy is a highly active area and there are studies reporting the targeting of opioid receptors to control cell growth and replication in colon cancer (Hytrek et al., 1996; Zagon, 1996).

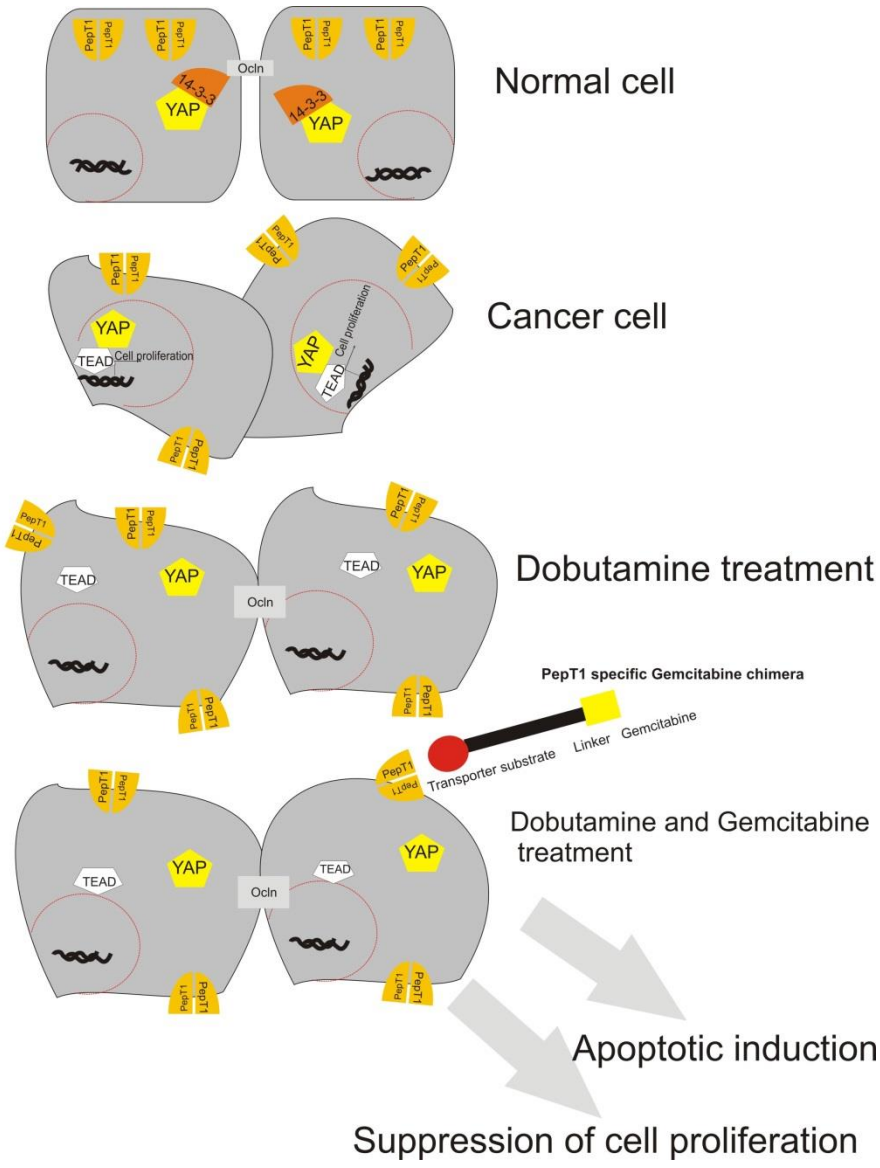

Figure 5.18 Diagram representing the potential adjuvant actions of dobutamine treatment of pancreatic cancer cell lines.

Chapter 6

6 General discussion and future work

6.1 General discussion and future work

Currently, chemotherapy continues to represent the preferred therapeutic option for most malignancies. Severe side effects and inefficient delivery to the tumour site, however, are still the main hurdles that limit the effectiveness of this treatment option. Particularly in pancreatic cancer, conventional and targeted therapies have largely failed to prolong survival and decrease mortality rates. Indeed, no current treatment option has shown long-term benefit to treat patients with advanced metastatic disease, which accounts for the majority of pancreatic cancer cases (80 %)(Hotz et al., 2007). Thus, there is an urgent need to find better-targeted therapies to pancreatic cancer.

The role of epithelial cell de-differentiation in improving current chemotherapies applied to pancreatic cancer was investigated in this thesis. Our work started by exploring the potential use of oligopeptide transporters expressed in pancreatic cancer cells as drug delivery targets. Following this, the interplay between the Hpo pathway signaling and TJ network was investigated in the context of cell proliferation suppression coordinated with apical-basal polarity completion. These studies concluded by elucidating the potential use of a β-adrenergic agonist drug, dobutamine, in the treatment of pancreatic cancer.

Chapter 3 outlined the potential use of PepT1 as a drug delivery target to treat CP and PDAC. In mammalian cells, the transport of amino acids across the plasma membrane is an essential physiological process that has been extensively studied. Drug transporters opened up a new arena of cancer cell targeting opportunities. In particular, PepT1 was found to be overexpressed in cancer tissue, including in pancreatic cancer, providing a promising new targeting strategy for the delivery of anti-cancer therapeutics. PepT1, normally restricted to the apical surfaces in polarised intestinal epithelial cells, was found to be distributed at the cell membrane of non-polarised cancerous cells and at the basolateral surface of ductal cells in CP biopsies. Further, inhibition of the amino acid

L-transporter system, also shown to affect the transport of oligopeptides through PepT1, suppressed the proliferation of human pancreatic cancer cells expressing PepT1 in a more pronounced manner compared to the cell line with reduced levels of PepT1 expression. These results shed light on the idea that PepT1 is required by AsPc-1 cells to divide and proliferate, highlighting the potential in targeting this system to inhibit pancreatic cancer progression.

Conjugation of potent anti-inflammatory molecules, like ibuprofen or the anti-cancer drug gemcitabine, to dipeptide analogues specific for the oligopeptide transporter, made these complexes into PepT1 substrates resulting in an enhanced uptake by pancreatic cancer cell lines. Quite remarkable was PepT1 expression in tissue from patients diagnosed with CP. Despite controversy, there seems to be a relationship between CP and pancreatic cancer. Detection of PepT1 in pancreatitis biopsies may provide a novel way to target inflamed pancreas prior to malignant transformation. This work has also highlighted a potential new way of specifically targeting anti-cancer drugs to tumour cells increasing clinical efficiency and reducing side-effects.

As for future work, further studies about transporters' overexpression in cancer stem cells may provide new insights into new targets for cancer chemotherapy. Given their importance in cancer biology and chemotherapy, translational research of drug transporters assumes a pivotal role. Moving towards an era of personalized cancer therapy, the inclusion of the wide variety of human drug transporter proteins into individual genetic screens would then allow for an optimization and individualized use of specific cancer medicines.

Oncologists are currently seeking to "tailor therapies" to better target and treat specific malignancy. The fundamental goal is to develop new therapeutic strategies that exploit unique biological tumour properties to increase their sensitivity to the modulation of a

specific cell-signaling pathway. Hpo pathway signaling mediated by YAP represents a newly delineated opportunity for therapeutic intervention as it is frequently deregulated in a variety of cancers. Hpo signaling pathway regulates planar cell polarity and thus modulates cell proliferation within epithelial cell sheets. As Chapter 4 presented, it was hypothesised that a plasma membrane component involved in cell-cell contacts, Ocln, could function as a sensor of apical-basal epithelial cell polarity with proliferation control through the Hpo pathway. To address this, interactions between Ocln and Hpo pathway elements were examined. Previously shown to bind to and be regulated by the non-receptor tyrosine kinase c-Yes, Ocln was shown here to also bind to the Hpo pathway element, YAP, and its co-activating element TEAD.

Given the widespread involvement of YAP in tumours from different origins, the distribution of Hpo pathway signaling elements as well as Ocln was evaluated in human biopsies from patients diagnosed with CP and PDAC. Ocln, c-Yes, YAP, and TEAD localisation in epithelial cells from normal pancreas tissue were more similar to the distribution of these proteins in HPAFII cells, while the distribution of these proteins in CP and PDAC biopsies was more consistent with the distribution pattern observed in AsPc-1 cells. In addition, it was also shown that pharmacological attenuation of YAP using dobutamine to alter TEAD-dependent transcription, increased functional TJ structures and diminished growth rates of HPAFII and AsPc-1 cells. Importantly, dobutamine treatment of HPAFII cells resulted in a striking loss of the transcription factor TEAD. Generally, these results suggested an additional link between Hpo and signals proceeding from the TJ network involving Ocln that may regulate proliferation in a manner that could affect epithelial cell homeostasis and oncogenesis.

As shown earlier, proteins from the polarity complex are suggested to interact with Hpo elements and negatively regulate YAP by altering its subcellular location. ZO-2, AMOT and YAP interactions have been described (Oka et al., 2012); this work has shown

co-localisation between Ocln and Hpo pathway elements such as YAP. As ZO-2 and AMOT proteins are localised at TJs (Guillemot et al., 2008), it cannot be discarded that YAP might be co-localised with Ocln through the interaction with ZO-2 and AMOT. More studies will need to address whether YAP directly binds to Ocln.

Stabilization of cell-cell contacts restores abnormal epithelial cell polarity representing an interesting therapeutic strategy in cancer. Despite the molecular mechanisms involved in the Hpo pathway and the YAP/TAZ-TEAD being clearly established, the activators, the upstream regulators and how these elements regulate tumour progression through Hpo signaling remain to be elucidated. Still, the efficacy of YAP inhibitors and low toxicity on normal tissues is also subject to further investigation. A more advanced understanding on how cells generate, receive and transmit Hpo signals will give further insights into the molecular paradigms for tumourigenesis.

In Chapter 5, the cytotoxic actions of dobutamine on pancreatic cancer cell viability and proliferation were confirmed. Dobutamine was shown to elicit an apoptotic response in AsPc-1 and HPAFII cells detected through nucleosome quantification. Given the role of proteasome inhibitors in inducing apoptosis in various cancer cells (Wente et al., 2005), it would be interesting to explore if the mechanism by which dobutamine reduces YAP and TEAD levels is mediated through a proteasome-mediated degradation of these proteins. If so, suppression of proteasome activity should block dobutamine actions. The compound MG-132 has been suggested to be a specific, potent, reversible, and cell-permeable proteasome inhibitor, reducing the degradation of ubiquitin-conjugated proteins in mammalian cells (Jensen et al., 1995). Thus, it would be interesting to examine the effect of MG-132 treatment on AsPc-1 and HPAFII cell proliferation and polarisation, cytotoxicity and apoptosis in the presence and absence of dobutamine.

General discussion and future work

The suppression of cell proliferation via dobutamine in AsPc-1 cells was suggested to be β-adrenergic mediated, although a more exhaustive experimental setup needs to be carried out to fully address this outcome. Most β-agonist mediated actions go through adenylyl cyclase/cAMP/PKA pathways, so the effects of phosphodiesterase inhibitors, which might mimic or potentiate dobutamine, are of interest to this system, particularly in the distributions of Ocln, YAP, and TEAD, and the enhancement of TER.

Forskolin is the standard adenylyl cyclase activator and acts synergistically with receptor agonists in elevating endogenous levels of cyclic AMP (De Vries et al., 1988). To check if dobutamine is acting via cAMP pathways, this compound should mimic the effects of the β-adrenergic agonist in the pancreatic cancer cell lines used, or not if other pathways are involved. Another possible compound to use in these studies would be 3-isobutyl-1-methylxanthine, IBMX, a methylated xanthine derivative (Kashiwagi and Foley, 1982); as the standard phosphodiesterase inhibitor that acts upon raising intracellular cAMP, if β-adrenergic mediation would be involved, this compound would be expected to mimic dobutamine effects in this system.

This current work did not address if the dobutamine effect is being mediated through receptor activation or if it is a consequence of receptor down-regulation. Hence, considering the involvement of arrestin pathways might be of some value. Once bound, β-arrestins prevent G-protein coupling (Luttrell and Lefkowitz, 2002), so then this would block the actions of dobutamine in the system tested.

Dobutamine was suggested to affect paracellular permeability through its actions on Ocln expression and stabilization at the cell membrane. Transient knock-down of Ocln expression confirmed that the actions induced by dobutamine on HPAFII cells were mediated through this TJ element. This work has also observed an essential role for PKCζ in the formation of functional TJs where the loss of action of this kinase could not

be compensated by dobutamine. The fact that dobutamine could not enhance TER in the presence of PKCζ inhibitor would imply that PKCζ acts down-stream of dobutamine. As the ROCK inhibitor attenuates dobutamine effect, ROCK might also be placed down-stream of dobutamine. Therefore in the first scenario, it would be proposed that dobutamine acts to recruit Ocln to TJs via PKCζ and/or ROCK, and then Ocln inhibits YAP. Alternatively, dobutamine inhibits YAP and then the inhibition of YAP-dependent transcriptions and/or the physical interaction of YAP with Ocln might cause the redistribution of Ocln and the maturation of TJs. To test the first scenario, Ocln knockdown model and inhibitors of PKCζ and ROCK would block the dobutamine-induced YAP inhibition. To test this, nuclear localisation of YAP and mRNAs of YAP target genes such as CTGF could be used as read-outs. If the second scenario is true, YAP knockdown should facilitate the redistribution of Ocln and enhance TER. Dominant active YAP mutant should block the effect of dobutamine on the redistribution of Ocln and the enhancement of TER. To follow up these studies, a collaboration has been established with Heidi Wilson´s group at the University of Colorado in Denver, whereby the mechanisms induced by dobutamine will be elucidated in an Ocln null mouse model. These studies will shed light on dobutamine regulation of downstream mechanisms in the absence of Ocln and will firmly establish the mechanism and clinical impact of dobutamine as an adjuvant therapy for pancreatic cancer.

In Chapter 5 it was also shown the potential in combining the cytotoxic actions of gemcitabine and dobutamine as an adjuvant chemotherapeutic approach to treat epithelial derived pancreatic cancer cells. PDAC, however, is characterized by an intense desmoplastic reaction composed of epithelial-derived cancer cells that are intimately associated with fibroblasts, macrophage, stellate cells, etc. It is perceived that a major challenge to effectively treat PDAC is the resistance of this desmoplastic environment to chemotherapeutics (Li et al., 2012). As shown, dobutamine was able to suppress proliferation of pancreatic epithelial cancer cells through the down-regulation of YAP and

TEAD resulting from Ocln-Ocln interactions; however this benefit might be nullified by the presence of non-epithelial cells that are commonly present in desmoplastic pancreatic tumours. Thus, the effect of desmoplastic components on dobutamine actions would address the usefulness in using this compound to target PDAC.

Although dobutamine was not tested in an *in vivo* model, this work highlighted a novel pharmacological approach that targets the Hpo pathway by potentially disrupting transcriptionally active complexes. Instead of inhibiting exclusively enzymatic activities or ligand-receptor interactions, this work might have opened new avenues into a possible new era of anti-cancer drugs: those that by interfering with the formation of complexes regulate the transcriptional machinery of a growth regulatory pathway. Importantly, being a drug that acts by increasing the cardiac output, studies need to evaluate carefully the potential benefit /risk ratio in using dobutamine as an adjuvant therapy to treat pancreatic cancer. Since smoking is indeed the most common cause of pancreatic cancer, these patients are therefore likely to carry a considerable risk of heart attack. Allied to this, many patients receiving chemotherapy would also be taking cardiovascular medications.

This work speculated about the activity of dobutamine in sensitizing pancreatic cancer cells to treatment with gemcitabine. Important to this discussion is also the translation of pre-clinical data into the clinic as these studies were performed in two pancreatic cancer cells line which are likely to display marked heterogeneity in sensitivity to gemcitabine. To fully address the potential of these findings, gene expression profiling and other analytical strategies should be employed to allow determining more precisely whether cell lines recapitulate the biological heterogeneity present in pancreatic tumours. Moreover, these findings also require comparisons of tumour biological properties in cells cultured in different ways: 2-D and 3-D culture *in vitro* and in subcutaneous and orthotopic xenografts derived from these cells.

Chapter 6

General discussion and future work

Overall, cancer therapies still strive to identify an effective targeted drug delivery system that reaches primarily the tumour site and sparse the adjoining tissues and organs. An infallible drug delivery method, however, is still beyond our grasp. To achieve this desired outcome, it is essential that pre-clinical models become better predictors of clinical activity allowing for an effective drug screen improving safety and efficacy outcomes in patients.

Chapter 7

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Chapter 7

Bibliography

Chapter 8

8 Appendix

A.1 – Supplementary figures

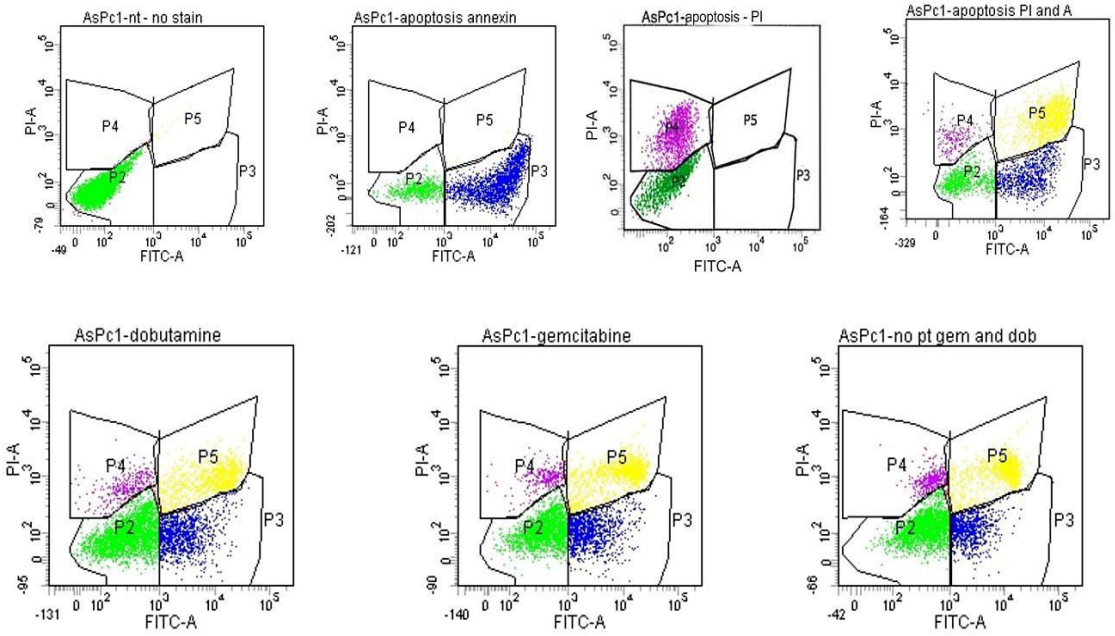

Figure 8.1 Controls used for the annexin/PI staining technique in AsPc-1 cells. Refers to Figure 5.13-A: Chapter 5. ***Abbreviations: nt – non-treated; no pt – no pre-treatment***

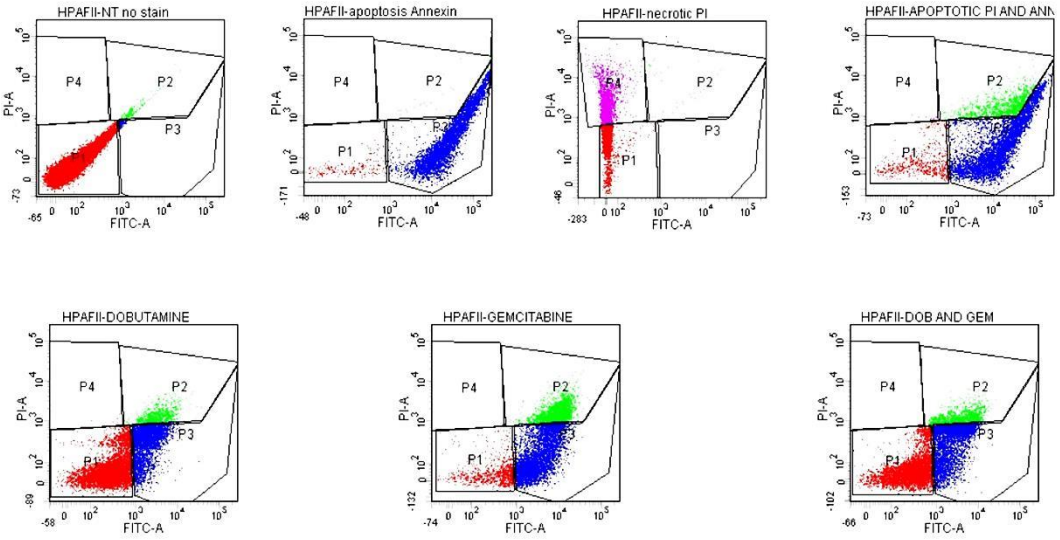

Figure 8.2 Controls used for the annexin/PI staining technique in HPAFII cells. Refers to Figure 5.13-B: Chapter 5.***Abbreviations: NT – non-treated;*** Cells in each quadrant can be interpreted in the following manner: **Lower left quadrant – P1**: These cells are negative for both annexin V and PI and can be regarded as viable non-apoptotic cells; **Lower right quadrant – P3:** Cells are positive for annexin V but negative for PI. These are cells that are at an early stage of apoptosis as the cell membrane remains intact and annexin V binds to phosphatidyl residues located in the outer membrane. **Upper left quadrant – P4**: Cells are negative for annexin V but positive for PI. These are cells that are at an end-stage of either apoptosis or necrosis and have lost their cell membrane or are dead. **Upper right quadrant – P5**: These cells are positive for both annexin V and PI. Although the cells show evidence of phosphatidyl residues these are not necessarily on the cell surface.

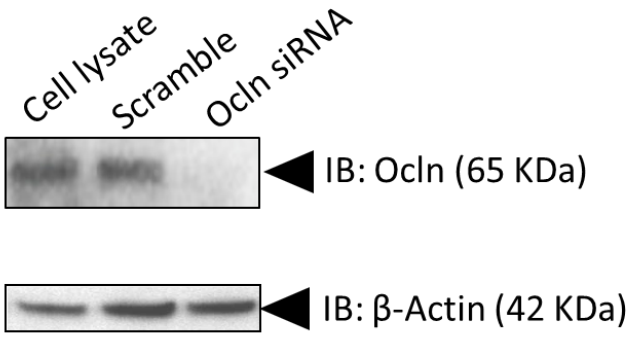

Figure 8.3 Immunoblot confirming Ocln knock-down in HPAFII cells. Refers to Figure 5.7-B: Chapter 5.

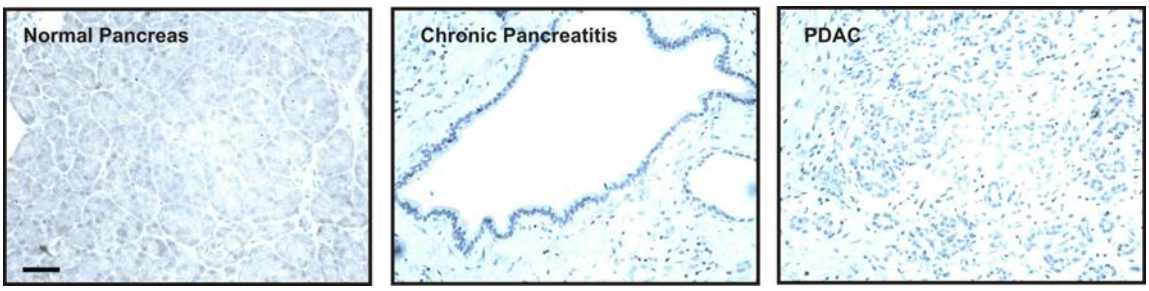

Figure 8.4 IHC controls for normal, chronic pancreatitis and pancreatic cancer human biopsies. Tissue sections were blocked for 30 min at RT with serum blocking reagent as described in section 2.10, Chapter 2 and incubated with a rabbit non-immune immunoglobulin (rabbit - DA1E; Cell Signalling) with the same concentration as the primary monoclonal antibody (1:100 dilution). Tissue sample was then incubated with the secondary antibody and detection reagents. Scale bar, 50 µm.

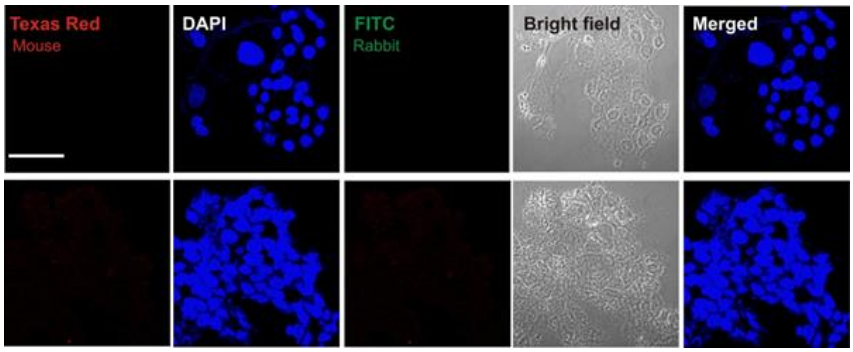

Figure 8.5 Immunofluorescence controls. No primary antibody controls for AsPc-1 (Top panel) and HPAFII (Bottom panel) cells. Confocal images of indicated fluorescent secondary antibodies (Mouse -Texas Red and Rabbit -FITC). Cells were stained as outlined in section 2.9 Chapter 2, with the exception that no primary antibody was added. Images are representative of three independent experiments; Scale bar, 20 µm.

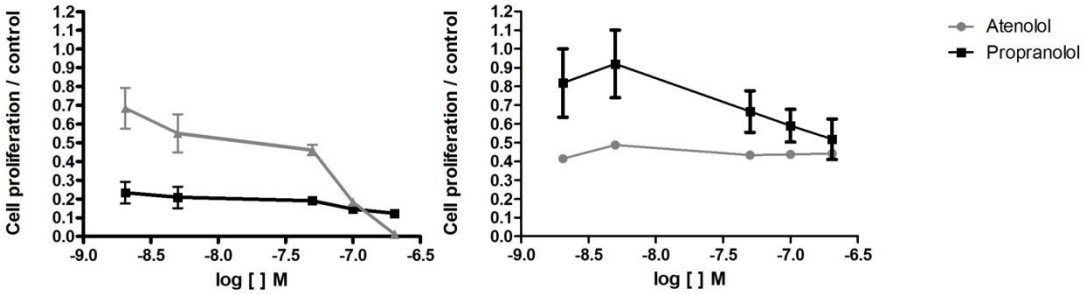

Figure 8.6 Cell proliferation in AsPc-1 (left hand side) and HPAFII (right hand side) cells in response to different concentrations of antagonists, atenolol and propranolol during 48 h. Refers to Figure 5.3, Chapter 2.

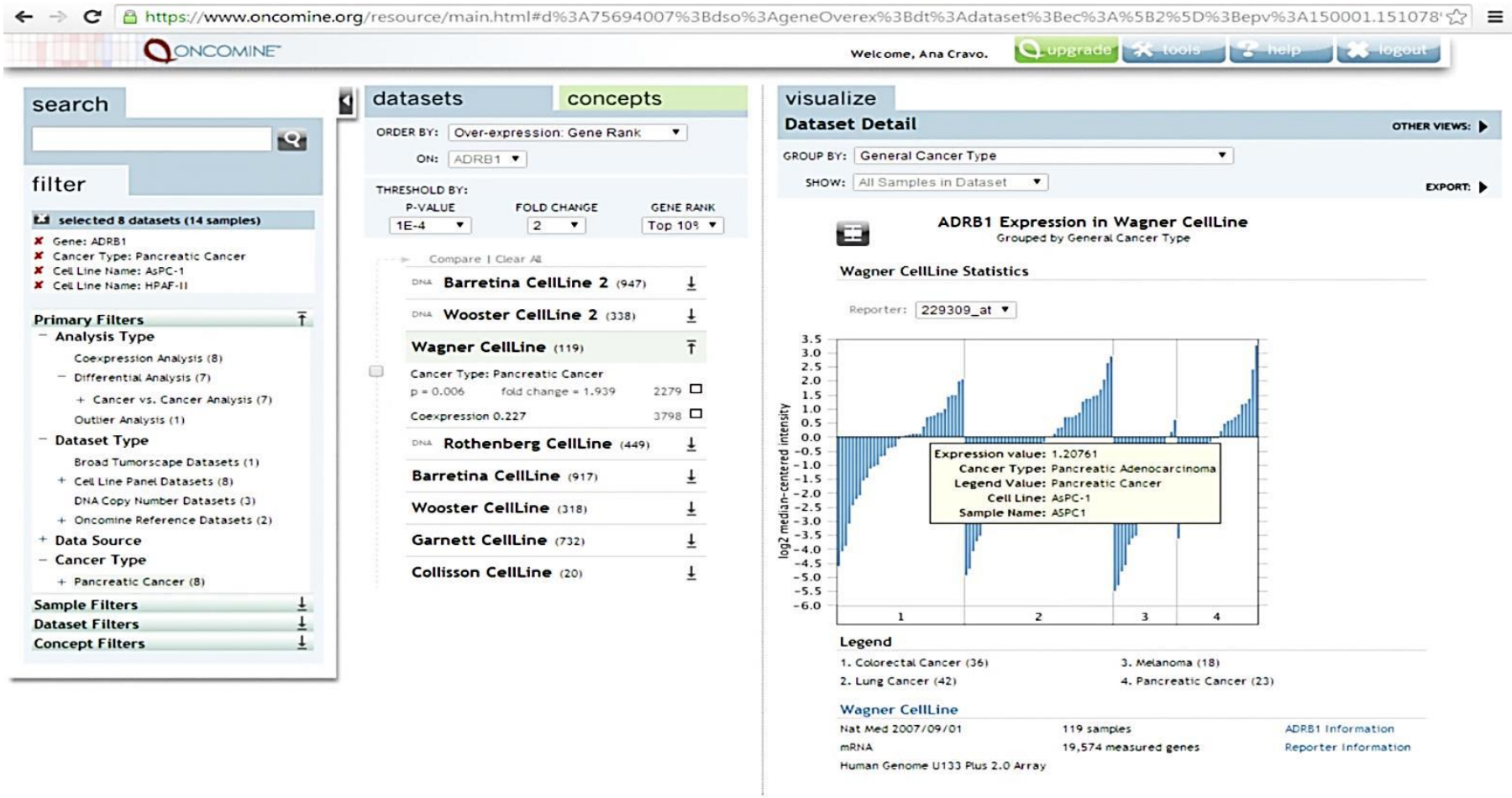

Figure 8.7 Adrenergic receptor β-1 expression in AsPc-1 cell line (expression value = 1.20761). Refers to Figure 5.3: Chapter 5.

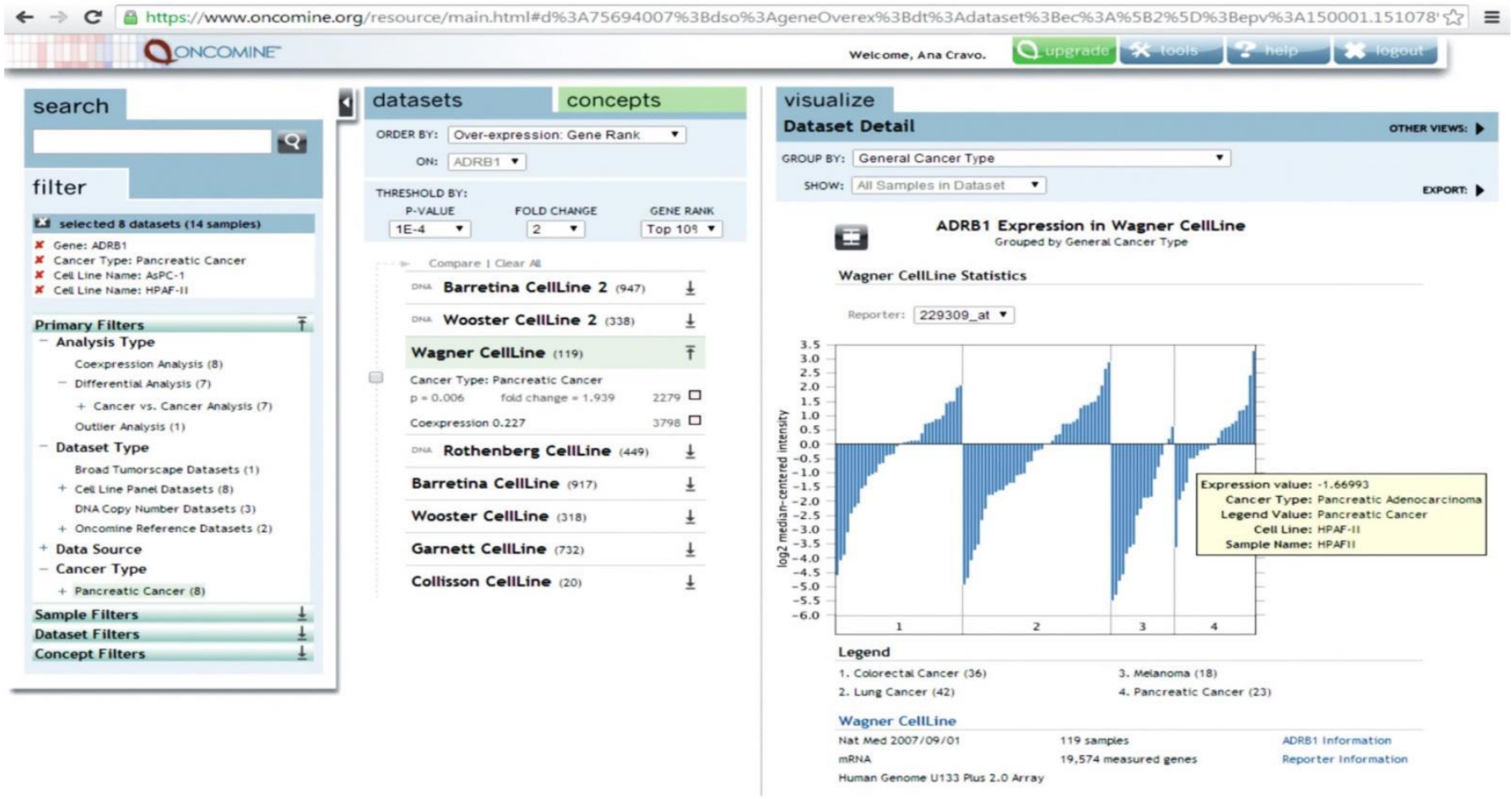

Figure 8.8 Adrenergic receptor β-1 expression in HPAFII cell line (expression value = -1.66993). Refers to Figure 5.3: Chapter 5.

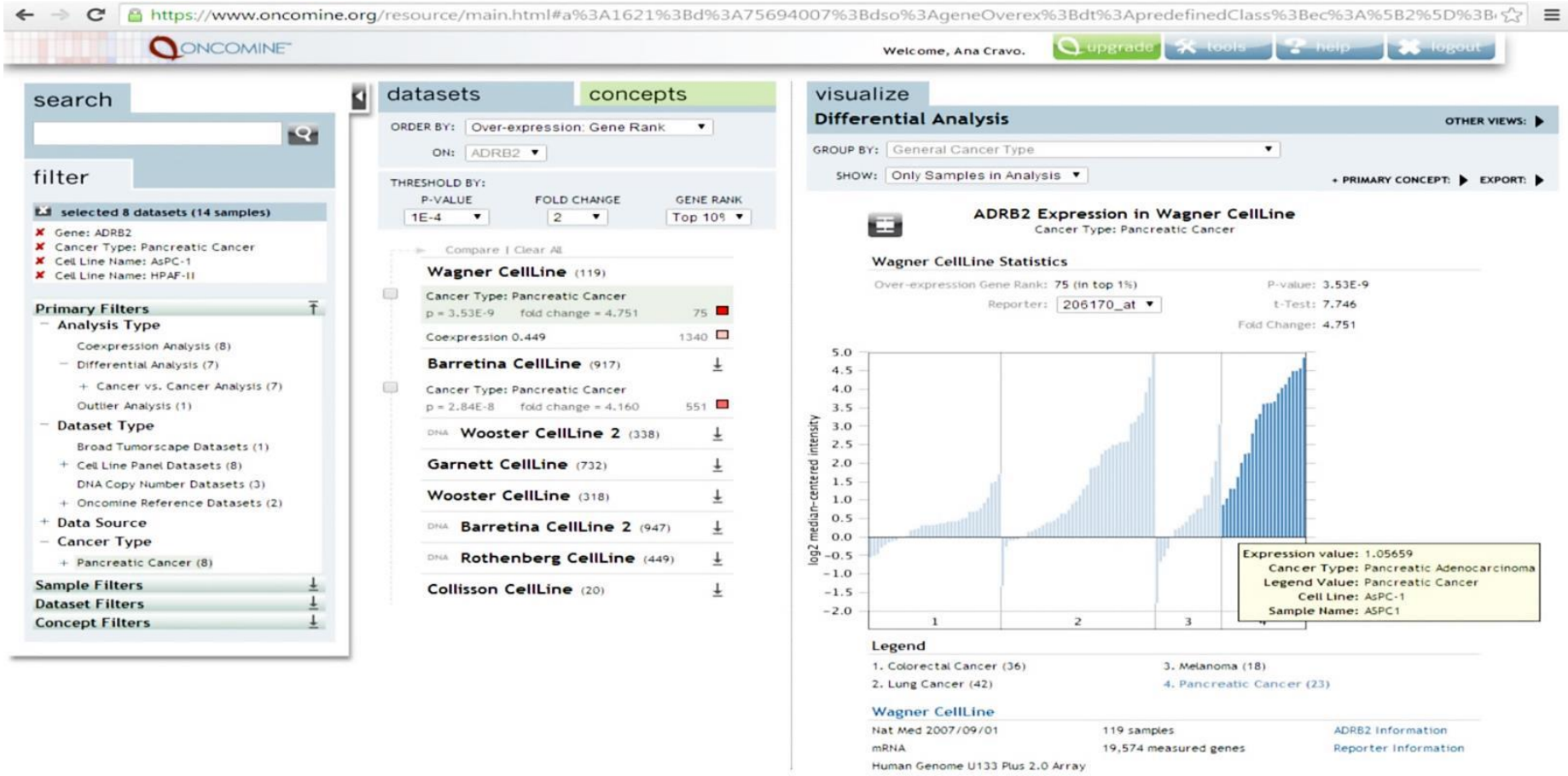

Figure 8.9 Adrenergic receptor β-2 expression in AsPc-1 cell line (expression value = 1.05659). Refers to Figure 5.3, Chapter 5.

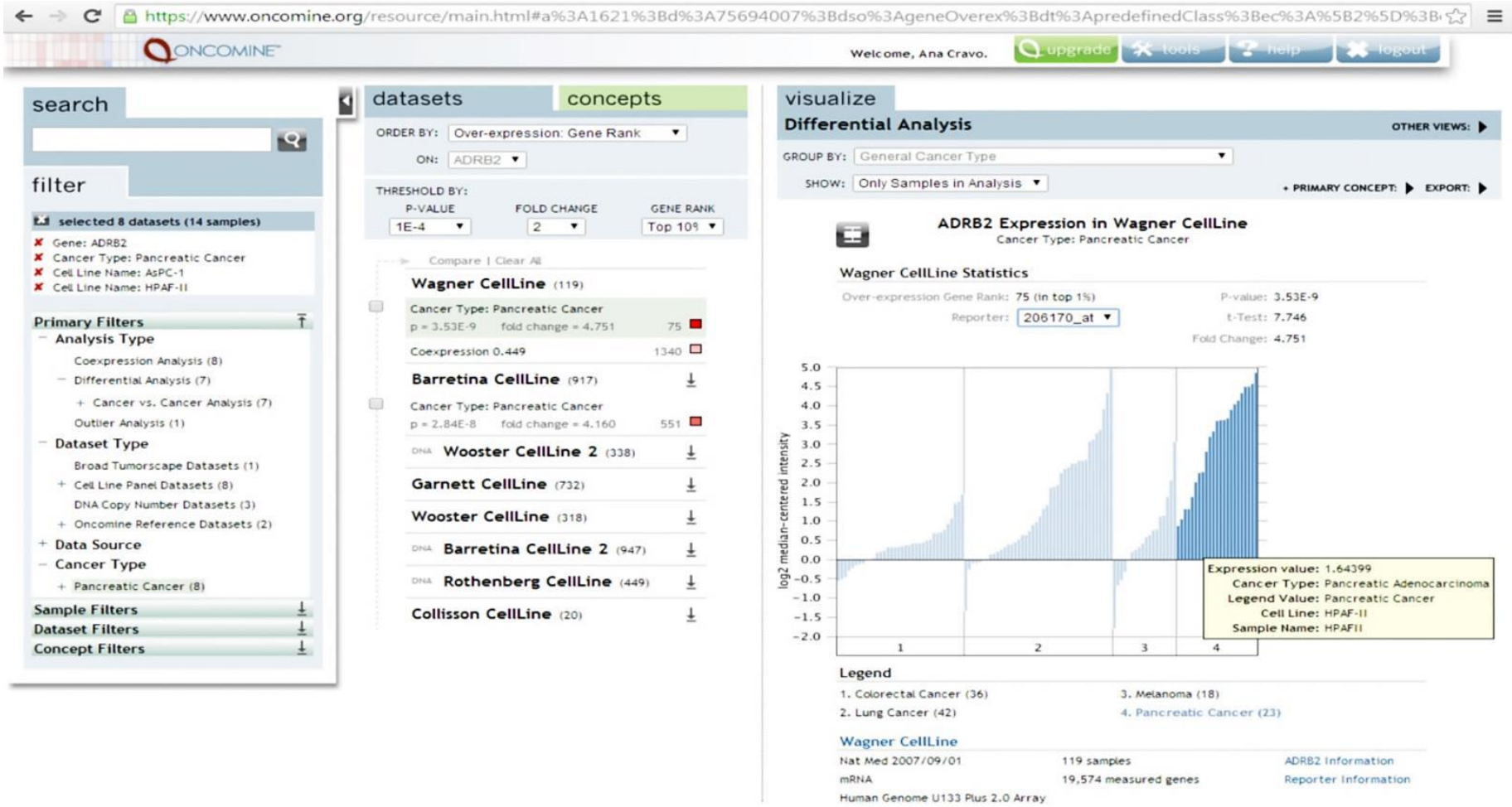

Figure 8.10 Adrenergic receptor β-2 expression in HPAFII cell line (expression value = 1.64399). Refers to Figure 5.3, Chapter 5.

Chapter 9

9 Dissemination

Chapter 9

Dissemination

Posters and Oral presentations

● **26th June, 2012, Poster Presentation at the Graduate Research Afternoon, University of Bath;**
Title: **Targeting and delivering to cancer cells through the transporter system PepT1**

● **28th June, 2012, Oral Presentation at the Meeting of Minds, University of Bath;**
Title: **Targeting and delivering to cancer cells through the transporter system PepT1**

● **14th November, 2012, Poster Presentation at the Department of Pharmacy and Pharmacology, University of Bath;**
Conference: Cancer Research at Bath Symposium
Title: **Targeting and delivering to pancreatic cancer cells through the transporter system hPepT1**

● **29th September, 2012, Poster Presentation at Royal Society of Medicine, London;**
Conference: Development of cancer clinical medicines using in vivo models.
Title: **Targeting and delivering to pancreatic cancer cells through the transporter system hPepT1**

● **24th January, 2013, Poster Presentation in the European Organisation for Research and Treatment of Cancer, Cardiff;**
Conference: 34th EORTC-PAMM - BACR Winter Meeting
Title: **Targeting pancreatic cancer through the oligopeptide transporter PepT1**

● **4th May, 2013, Poster Presentation at the Cancer Research UK Cambridge Institute, University of Cambridge;**
Conference: Pancreatic Cancer Symposium
Title: **PepT1- A potential strategy to target chronic pancreatitis and pancreatic cancer.**

● **3rd July, 2013, Poster Presentation at University of Bristol;**
Conference: Tumour Microenvironment
Title: **PepT1 – a potential strategy to treat chronic pancreatitis and pancreatic cancer**

● **3rd September, 2013, Oral Presentation at the Heriot-Watt University, Edinburgh UK;**
Conference: PharmSci, 2013
Title: **PepT1 – A potential strategy to target therapies for chronic pancreatitis and pancreatic cancer**

● **16th December, 2013, Oral Presentation at the Department of Pharmacy and Pharmacology, University of Bath;**
Conference: Departmental Research Afternoon
Title: **PepT1 – A potential strategy to target therapies for chronic pancreatitis and pancreatic cancer**

Awards

● **June, 2013, 2nd best image of research voted by general public**
Event: Images of Research Competition, University of Bath
Image´s title: Expression of the nutrient uptake transporter system PepT1 in human chronic pancreatitis;
http://www.bath.ac.uk/research/images-of-research/

● **23rd, June, 2014, Image of research selected biomedical picture of the day**
Event: Biomedical picture of the day, MRC
Image´s title: Hijacked transporters;
http://bpod.mrc.ac.uk/archive/2014/4/6

Grants

This work served as the basis for two different grants:

Project title: Alteration of Hippo Signalling to Increase Sensitivity of Pancreatic Cancer cells to Gemcitabine;
Pancreatic Cancer Research Fund
Pancreatic Cancer UK

Publications

Book chapter

Published
Cravo, A., and Mrsny, R. (2013). A Time Travel Journey Through Cancer Therapies. In Cancer Targeted Drug Delivery, Y.H. Bae, R.J. Mrsny, and K. Park, eds. (Springer New York), pp. 3-35.

Journal articles

Submitted

Cravo A, Carter E, Erkan M, Furutani-Seiki M, Harvey E, Mrsny R (2014)
Occludin interacts with YAP to Connect Tight Junction Status with Hippo Pathway Signalling

In preparation

Cravo, A., Wilson, G., Erkan, M., Bailey, P., Mrsny, R., (2014) PepT1 as a Drug Delivery Target to Treat Chronic Pancreatitis and Pancreatic cancer.